

Archives of Oral Biology

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FOREWORD

Growth of research in the many fields which have significance for the advance of knowledge in oral biology has been such that existing channels of publication have been overburdened. Furthermore, much work of greatest interest in this special field has perforce appeared scattered in scientific journals of many kinds from many lands where it does not quickly reach the notice of those most able to assess its value. The production of acceptable original papers is not now confined to a few sources or a few countries and speedy publication in one of the languages in international use has become an important requirement. These considerations have led to the publication of the *Archives of Oral Biology* conducted with the assistance and co-operation of leading workers in all parts of the world. Its scope will be catholic, but it will extend a particular welcome to fundamental studies making use of any of the disciplines of science, both by established methods and by new techniques which in many cases await application to oral biology. In this field more than in most branches of the biological and medical sciences, the development of a strong body of research workers is recent and in some parts of the world hardly yet achieved and fraught with difficulty. To these workers the *Archives of Oral Biology* presents at once the means of integrating their work with the most recent findings of others and of promoting that international co-operation in science which we all desire. It presents a challenge which we believe every centre of learning will wish to accept.

F. A. ARNOLD
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A SURVEY OF THE BACTERIAL FLORA OF THE PERIODONTIUM IN THE RICE RAT*

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Abstract—A survey of the bacteria recoverable from the periodontium of normal rice rats and rice rats with periodontal destruction was undertaken. Forty-five strains of bacteria isolated from fourteen rice rats were examined by appropriate tests to permit generic classification. Recurring strains included enterococci, coli-aerogenes species, *Actinomyces*, *Lactobacilli*, aerobic diphtheroids, *Staphylococci*, and possibly *Actinobacillus* species. *Bacteroides* and *Fusobacterium* were recovered infrequently and spirochaetes were never seen.

STUDIES of the bacterial flora of the gingival pocket in man suggest that inflammatory and degenerative disturbances of the periodontium are associated with increases in numbers of the resident flora (HEMMENS and HARRISON, 1942; ROSEBURY, MACDONALD and CLARK, 1950). Aetiological significance in periodontal diseases has been attributed to the increase as a whole (ROSEBURY *et al.*, 1950) or to increase of particular components of the flora (SCHULTZ-HAUDT and SCHERP, 1955). Very little is known about the flora of the periodontium in animals in relation to periodontal disease, although several species have been reported to be subject to disturbances of the periodontium. Indeed, remarkably few attempts have been made to characterize the total oral microbiota of experimental animals. ROSEBURY and KARSHAN (1931) and ROSEBURY, FOLEY and GREENBERG (1934) showed that lactobacilli and *Escherichia coli* were regularly present in the mouths of laboratory rats. HARRISON (1940) investigated the aerobic flora of the rat and found that streptococci were more numerous than the total of all other cultivated species. The latter consisted of various staphylococci, coliforms and other organisms identified only morphologically. Monilia and filamentous forms were occasionally recovered. ROGOSA (1956) cultivated *Veillonella* species from the oral cavities of 59 per cent of 545 rats. Diphtheroids have been demonstrated to be present regularly as a component of the rat oral flora (ROGOSA, SHIOTA and DISRAELY, 1957). ORLAND (1946) in a study concerned primarily with aciduric organisms in the hamster oral cavity found that in addition to lactobacilli, streptococci, staphylococci and unidentified pleomorphic organisms growing in acid media, a wide variety of micro-organisms grew on blood agar plates. MITCHELL and JOHNSON (1956) examined the gingival plaque in hamsters by stained smears and found streptococci, staphylococci and various pleomorphic organisms in addition to organisms tentatively identified by cultural techniques as lactobacilli. ROGOSA,

* Supported in part by a research grant, D-579, from the National Institute of Dental Research.

JOHANSEN and DISRAELY (1957) in a report concerned principally with numbers of streptococci and lactobacilli in the hamster oral cavity noted that Gram stained smears frequently revealed streptococci, fusiforms, yeasts and very large pleomorphic coccoid organisms. Lactobacilli and spirochaetes were occasionally observed.

Recent studies by GUPTA and SHAW (1956) suggest that periodontal disease in the rice rat is comparable histopathologically to periodontitis in man. The present study is one of a series dealing with the bacteriology of the periodontium in the rice rat and its relation to periodontal disease. Reported here are investigations of the types of organisms found in animals with normal gingivae or with periodontal disease.

METHODS AND MATERIALS

Fourteen 21 week old rice rats, unselected in respect to sex and maintained on diet 700 (GUPTA and SHAW, 1956) with or without added B vitamins, were sacrificed with chloroform, decapitated and the skins removed from their heads. Five of these animals had grossly normal periodontal structures and nine of them had evident periodontal disease. Using aseptic technique the heads were sectioned sagittally and the molar teeth and associated periodontal structures of the right side were removed *en masse* with sterile Rongeur forceps. The teeth and periodontium were suspended in 2 ml of heart infusion broth and ground in a glass tissue grinder. Samples of the resulting suspensions were examined by dark-field illumination. In three instances, dark-field examination was made also of samples taken directly from the gingival crevice before removing the teeth from the jaws. Samples were then plated aerobically and anaerobically on veal heart infusion horse blood agar, and anaerobically in lactate agar (ROGOSA, 1956). The latter were prepared as pour plates for the purpose of disclosing *Veillonella* species. All plates were incubated at 37°C, the anaerobic plates for 5-7 days, the aerobic plates for 3 days. For anaerobic incubation, cultures were placed in Brewer jars and incubated in 95 per cent H₂, 5 per cent CO₂.

The plates were examined under a stereoscopic microscope and 137 colonies were examined by dark field and by Gram stain. From representative colonies, forty-five pure cultures were derived by passage on blood agar and in heart infusion broth. For purposes of further study, the forty-five strains were divided on the basis of Gram stain, cellular morphology and aerobic or anaerobic growth into the following categories: streptococci, Gram-positive and Gram-negative aerobic bacilli, Gram-negative microaerophilic coccobacilli, *Bacteroides* and *Fusobacterium*, Gram-positive anaerobic bacilli. All strains were carried on heart infusion blood agar and in Difco thioglycollate broth and Difco brain heart infusion broth. In addition, all strains were tested for catalase production (KING and MEYER, 1957).

Streptococci were further examined for haemolysis on blood agar, growth in heart infusion broth at 10°C and 45°C, growth at pH 9.6, or with 6.5% sodium chloride, and in skimmed milk containing 0.1 per cent of methylene blue. They were also examined for the production of mucoid colonies on 5% sucrose agar.

Gram-positive aerobic bacilli were examined initially for catalase production, indole production, and spore formation (MACDONALD, 1953). Non-sporulating aerobic Gram-positive bacilli which were catalase and indole negative were grown

on Difco serum tellurite agar and Difco Rogosa SL agar to distinguish between diphtheroids and lactobacilli. Additional examinations included tests for nitrate reduction (WILSON and MILES, 1957) and the production of lactic acid from glucose. The test for lactic acid was based on the method of BARKER and SUMMERSON (1941); lactic acid was converted to acetaldehyde, followed by condensation with *p*-phenylphenol. The test was found sensitive to 0.01% lactic acid under the conditions employed.

Gram-negative aerobic bacilli were grown on Difco E.M.B. agar and identified on this basis as coliforms or late lactose fermenters. The latter were tested for indole production and the Voges Proskauer and methyl red reactions. Examination of late lactose fermenters for the production of cytochrome oxidase was used as a test for a distinctive characteristic of *Pseudomonas* species (GABY and HADLEY, 1957).

Strains of Gram-negative coccobacilli about $0.5 \times 1.0 \mu$, sometimes accompanied by short filaments, initially microaerophilic, were subsequently cultivated readily both aerobically and anaerobically on nutrient agar. They were examined for catalase and indophenol oxidase production (GORDON and MCLEOD, 1928). Fermentations in BBL trypticase agar with various carbohydrates in 1, 3 and 6% concentrations were recorded after 21 days aerobic incubation. Carbohydrates used were mannitol, maltose, sucrose, galactose and glucose (GOLDSWORTHY, 1938; THJOTTA and SYDNES, 1951). The strains were cultured also in litmus milk.

Gram-positive anaerobic bacilli were tested first for spore formation. Non-spore-formers were examined for catalase production and the formation of lactic acid from glucose. Fermentation of xylose, salicin and raffinose was used as a basis for distinguishing between *Actinomyces* and anaerobic diphtheroids as suggested by KING and MEYER (1957).

Appropriate positive and negative controls were employed with all of the above tests.

RESULTS

The dark-field examinations revealed a flora composed mainly of cocci and non-motile rods. Fusiforms and motile rods were occasionally seen, but spirochaetes were never observed. By this method differences were not detected between the flora of animals with normal gingival tissues and those with periodontal disease.

Cultural and morphological data on the aerobic Gram-positive cocci indicated the recovery of both staphylococci* and streptococci. Catalase production by the former and not by the latter confirmed the distinction. Organisms classified as staphylococci were not examined further. The streptococci produced no haemolysis on blood agar, non-mucoid colonies on sucrose agar, grew at 10°C, at 45°C, at pH 9.6, in broth containing 6.5 per cent of sodium chloride and in skimmed milk containing 0.1 per cent of methylene blue. On the basis of these findings, they were identified as enterococci.

The aerobic Gram-positive bacilli, except for a single spore-forming strain, fell into two main groups. One group was catalase positive and produced black or grey colonies on serum tellurite agar and failed to grow on Rogosa agar; these were

*See footnote to Table 1, p. 5.

classed as aerobic diphtheroids. The second group, identified as lactobacilli, was catalase negative, grew on Rogosa agar but not on serum tellurite agar, did not produce indole or reduce nitrates, and produced lactic acid from glucose.

The aerobic Gram-negative rods included organisms producing colonies typical of *Esch. coli* on E.M.B. agar and also organisms forming late lactose fermenting colonies. The latter on blood agar produced confluent spreading growth accompanied by haemolysis. These strains were catalase and methyl red positive, indole and Voges Proskauer negative and failed to give evidence of cytochrome oxidase production. They appeared to fall into the category of paracolon bacilli.

The Gram-negative microaerophilic coccobacilli were found to be catalase negative and did not produce indophenol oxidase. On nutrient agar they produced small translucent colonies with a tendency to adhere to the medium. Fermentation reactions were variable. Glucose, sucrose, galactose and maltose were fermented by some strains, not by others. None fermented mannitol. None fermented any of the tested carbohydrates when present in 6% concentration. All strains grew in litmus milk and fermented it slightly. The identity of these organisms is uncertain. Morphologically and culturally they appear compatible with descriptions of the genus *Actinobacillus* and particularly *Actinobacillus actinomycetum-comitans*. Descriptions of this genus are few in number and species characteristics are uncertain (WILSON and MILES, 1957). On the basis of the limited data and more particularly on the negative grounds that the strains isolated in the present study do not appear to belong elsewhere, they are tentatively identified with the genus *Actinobacillus*.

Gram-negative anaerobic rods identified as *Bacteroides* varied from coccobacilli to filamentous pleomorphic forms. Strains showing tapered cells and producing iridescent, mottled smooth colonies on blood agar were found and classified as fusiforms.

The Gram-positive anaerobic rods were catalase negative and fermented xylose, salicin and raffinose, but did not form lactic acid, thus conforming to the characteristics of *Actinomyces* proposed by KING and MEYER (1957). A single strain of anaerobic Gram-positive spore-forming bacilli was presumed to be a *Clostridium*.

The distribution of the above types among the forty-five strains is shown in Table 1.

DISCUSSION

The present study appears to be the first attempt at a comprehensive survey of the bacteria recoverable from the oral cavity of an experimental animal. The study is qualitative and was undertaken as a prerequisite for quantitative studies to be reported later. The utilization of several media and a broad spectrum of judiciously selected biochemical tests made it possible to identify the strains isolated with reasonable confidence. It should be noted, however, that the methods took no account of the possible occurrence of fungi, protozoa or pleuropneumonia-like organisms, and obviously ignored any resident viruses or rickettsiae. Subject to these limitations the findings can be considered an adequate survey of the spectrum of bacteria recoverable from the oral cavity of the rice rat.

TABLE 1. THE DISTRIBUTION OF FORTY-FIVE BACTERIAL STRAINS ISOLATED FROM THE PERIODONTIUM OF RICE RATS

Organisms	Number of strains
Enterococci	13
Paracolon bacilli	9
<i>Actinomyces</i>	6
Aerobic diphtheroids	4
Aerobic lactobacilli	4
<i>Actinobacillus</i> (?)	3
Coliforms	2
<i>Bacteroides</i>	1
<i>Fusobacterium</i>	1
<i>Clostridium</i>	1
<i>Bacillus</i>	1
<i>Staphylococci</i>	*

* *Staphylococci* were not among the forty-five strains examined but were disclosed sporadically, sometimes in large numbers, in subsequent quantitative studies. They grew facultatively on nutrient agar and produced large creamy-white colonies.

The flora, in general terms, can be described as predominantly aerobic and Gram-positive. Of the forty-five strains examined thirty-six grew aerobically; twenty-nine strains were Gram-positive. These figures cannot be interpreted as necessarily representing the proportion in which organisms occurred in the oral cavity but, since no conscious effort was made to select particular colony types, it is reasonable to conclude that the types predominating in this survey were probably the most numerous of the cultivable species. Support for this view was derived by applying the replica technique of LEDERBERG and LEDERBERG (1952) to blood agar plates inoculated with rice rat flora and incubated anaerobically. Velveteen impressions of growth on these plates were transferred to aerobic blood agar (SUZUKI, 1956) and the resultant colonies provided a measure of the proportion of total facultative and anaerobic growth which would grow aerobically. The counts indicated that approximately 75 per cent of the total was composed of facultative organisms. More than half of the isolated strains were enteric organisms—thirteen enterococci and eleven coliforms. This finding is not surprising in view of the coprophagic habits of rodents.

A striking finding was the complete absence of spirochaetes in this survey. These organisms, if present at all, would not easily be missed in the number of dark-field examinations conducted. It seemed of interest to examine the intestinal flora of the rice rat for spirochaetes, and this was done by dark-field examinations of three animals with completely negative findings. Evidently some ecological factor in these animals prevents spirochaetes from establishing themselves in the gastro-intestinal tract. PARR (1923) demonstrated spirochaetes regularly in the intestinal tract of man, laboratory rats and mice, and guinea-pigs. He found spirochaetes in only 21 per cent of wild rats, 22 per cent of wild mice and in none of thirty-seven rabbits. ROSENTHAL, McNABB and SNYDER (1939) failed to find spirochaetes in a variety of experimental

animals, and reported further that certain animal salivas have the property of promptly immobilizing oral spirochaetes of man.

An additional notable contrast between the rice rat and human oral floras was the relatively rare recovery of *Bacteroides* or *Fusobacterium* from the former. A single strain of each of these genera was isolated, whereas in man species of these organisms are regularly found in considerable numbers (ROSEBURY *et al.*, 1950; OMATA and DISRAELY, 1956).

It is difficult to compare the findings in the rice rat with those in other experimental animals because the data are limited. The aerobic flora of the laboratory rat, in so far as it has been investigated, seems similar to that of the rice rat (HARRISON, 1940; ROGOSA, SHIOTA and DISRAELY, 1957), with the exception of occurrence in the latter of species tentatively identified with the genus *Actinobacillus*. The anaerobic flora of the two animal species appears to be distinguished by the frequent occurrence of *Veillonella* in the white rat (ROGOSA, SHIOTA and DISRAELY, 1957) and not in the rice rat. *Actinomyces*, *Fusobacterium* and *Bacteroides* were found in the present study but have not been recorded as present in the white rat oral cavity. This difference may reflect merely the fact that anaerobic cultivation has been employed rarely. The finding by ROGOSA, JOHANSEN and DISRAELY, (1957) of spirochaetes in the oral cavity of the hamster and their report of frequent observation of substantial numbers of fusiform bacteria contrast with the present findings. It is of some interest, however, that they described late lactose fermenting intermediates of the coli-aerogenes group as occurring in the faeces of hamsters—possibly comparable to the paracolon strains isolated here.

The difficulty of making useful comparisons of the flora in various animals draws attention to the dearth of studies of normal flora of the mouth and other mucous membranes. More basic surveys of the flora of experimental animals, particularly those used in studies of dental disease, are needed.

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VERSCHIEDENE METHODEN ZUR BESTIMMUNG DER PHOSPHATASEAKTIVITÄT IN RINDERZÄHNEN*

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Zusammenfassung—Für die quantitative Bestimmung der Phosphatase-Aktivität in Zähnen werden die günstigsten Aufarbeitungsmethoden angegeben. Mit den Werten, die auf Grund von 5 aus der Literatur bekannten, jedoch dem vorliegenden Zweck besonders angepassten Methoden gewonnen wurden, werden die mit einer neu beschriebenen Fluoreszenz-Methode erhaltenen Werte verglichen. Auf Grund einer einheitlichen Definition der Phosphatase-Einheit kann die Empfindlichkeit der geprüften 6 Methoden verglichen werden. Die Empfindlichkeit fällt in folgender Reihenfolge (Methode nach dem verwandten Substrat bezeichnet): Dinatrium-*p*-Nitrophenylphosphat, Dinatriumphenylphosphat, β -Methyloxycumarinphosphorsäure-ester, Dinatrium- β -glycerophosphat, Tetracoluminphenolphthaleinphosphat, *o*-Carboxylphenylphosphorsäure. Mit den beiden empfindlichsten Methoden wird der Versuch unternommen, in reinem Dentin und reinem Schmelz von Rinderzähnen eine Phosphatase-Aktivität zu bestimmen. Während im Dentin eine Phosphatase beträchtlicher Aktivität nachgewiesen werden kann, ist im Schmelz nicht die geringste Aktivität dieses Ferments nachzuweisen.

Abstract—The best methods are given for a quantitative determination of phosphatase activity in teeth. The values which were obtained on the basis of five methods known from the literature but especially suitable for the present purpose were compared with the values obtained by a recently described fluorescence method. The sensitivity of the tested six methods can be compared on the basis of a uniform definition of the phosphatase unit. The sensitivity falls into the following sequence (method described according to the related substrate): disodium-*p*-nitrophenylphosphate, disodium-phenylphosphate, β -methyloxycoumarinicphosphoric acid ester, disodium- β -glycerophosphate, tetracoluminphenolphthalein phosphate, *o*-carboxylphenylphosphoric acid. With the two most sensitive methods an attempt is made to determine a phosphatase activity in pure dentine and pure enamel from animal teeth. Whereas a phosphatase of considerable activity can be detected in dentine, not the slightest activity of this enzyme can be found in the enamel.

ZAHLEICHE Forscher haben sich mit dem Nachweis der alkalischen Phosphatase in Kalkgeweben befasst und dabei im Knochen chemische und histochemische, im Zahn im wesentlichen histo-chemische Methoden angewandt. Während über den Knochen zahlreiche, im grossen und ganzen übereinstimmende Untersuchungen vorliegen, so dass man über Verhalten und Eigenschaften der Knochenphosphatase recht gut unterrichtet ist, liegen über den Stoffwechsel der Zähne nur wenige Arbeiten vor. Die Ähnlichkeit in Stoffwechsel und Aufbau von verschiedenen

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Zahngewebe und Knochen spricht für die Wahrscheinlichkeit des Vorkommens von Phosphatase zumindest in Dentin und Zement, also im Zahn überhaupt. Die Angaben in der Literatur sind jedoch recht widerspruchsvoll. Über den Nachweis einer alkalischen Phosphatase in Zähnen berichteten ROBISON und SOAMES (1924), später auch MACKENZIE (1933), PROVISSONATO (1935), BERLINER (1936), ROCHE und MOURGUE (1942) und andere. PROVISSONATO gibt einige mit der β -Glycerophosphat-Methode erhaltene quantitative Werte für die Phosphatase-Aktivität in Menschenzähnen an (0220–0730 mg P pro g Zahn/6 Std.).

BERLINER zieht jedoch diese Ergebnisse stark in Zweifel, da er bei Nachprüfung der Versuche fand, dass die Empfindlichkeit der Bestimmungsmethoden nach JENNER und KAY (1931, 1932), BODANSKY (1932), FISKE und SUBBAROW (1925) für diesen Zweck unzureichend war. Er stellte fest, dass man auf ihrer Grundlage zwar qualitative, aber keine quantitativen Aussagen machen könne. WHITE und HESS (1956) finden in menschlichem Dentin keine lösliche, nur eine nicht extrahierbare Phosphatase. KREUDENSTEIN (1957) findet nur im Eluat aus Dentin-Schnitten von Rinderzähnen eine alkalische Phosphatase und schliesst daraus auf das Vorliegen eines Fermentes nur im Dentin-Liquor (KREUDENSTEIN und STÜBEN, 1956), nicht strukturgebunden im Gewebe selbst.

Als Erklärung dafür, dass einzelne Autoren eine Phosphatase im Zahn bzw. im Dentin nachweisen konnten, andere nicht, kommen folgende Möglichkeiten in Frage:

- (1) Nachweismethoden verschiedener Empfindlichkeit.
- (2) Verwendung verschiedener Puffer. So wird von J. BUCH und H. BUCH (1939) über eine Hemmung der Phosphatase durch Veronalpuffer, von GOMORI (1949) über eine Hemmung durch Boratpuffer, jedoch nur gegenüber bestimmten Substraten berichtet.
- (3) Inaktivierung der Phosphatase durch die bei der Zerkleinerung der Zähne schwer vermeidbare Wärmeentwicklung.
- (4) Artspezifische Unterschiede.

Zuverlässige Aussagen, ob im Zahnhartgewebe überhaupt eine extrahierbare Phosphatase vorkomme, waren nur zu erwarten, wenn es gelang, schonende Aufarbeitungsverfahren und empfindliche Nachweismethoden anzuwenden. Zunächst musste das Aufarbeitungsverfahren festgelegt werden. Hierbei wurde die Enzymaktivität mit der Dinatrium-Phenylphosphat-Methode (s. unten) verfolgt, weil mit dieser Methode schon längere Erfahrungen bestanden. Nachdem das Aufarbeitungsverfahren festgelegt war, wurde eine Reihe weiterer aus der Literatur bekannter Nachweismethoden für den vorliegenden Zweck so gestaltet, dass eine Vergleichsmöglichkeit gegeben war. Eine bisher in der Literatur noch nicht bekannte Methode wurde neu entwickelt. Diese beruht auf dem Freiwerden einer fluoreszierenden Substanz, Methyloxycumarin. Von dieser Methode wurde eine besonders hohe Empfindlichkeit sowie die Möglichkeit einer kontinuierlichen Messung der Fermentaktivität erwartet.

Zur Vereinfachung und Abkürzung des Aufarbeitungsverfahrens und dadurch bedingter Enzymverluste wurden die Untersuchungen am ganzen Zahn durchge-

führt. Um für alle Untersuchungen ausreichendes und möglichst homogenes Ausgangsmaterial zu haben, wurden Rinderzähne verwandt. An Schmelz und Dentin wurde lediglich ein, auf den Erfahrungen der Versuche an ganzen Zähnen basierender, abschliessender Versuch durchgeführt.

I. Aufarbeitung der Zähne, Gewinnung des Fermentextraktes

Die Extraktion organischen Materials, also auch der Phosphatase, muss um so erschöpfender sein, je kleiner die Korngrösse ist, je mehr anorganisches Material in Lösung geht und je länger die Extraktionszeit ist. Andererseits bestehen Gefahren der Zerstörung des Fermentes. Denn je intensiver die Zerkleinerung ist, um so stärker ist die Wärmeentwicklung. So ist nach Zerkleinerung in der Kugelmühle die Aktivität der Phosphatase erheblich herabgesetzt. (vgl. Tabelle I). Stoffe, die die anorganischen Bestandteile in Lösung bringen, greifen häufig auch das organische Material an. Da die Extraktion unter Schütteln vorgenommen wird, wird man mit einer Schüttelinaktivierung der Fermente zu rechnen haben. Somit mussten die einzelnen Vorgänge genau geprüft werden.

(1) *Einfluss der Zerkleinerung, günstigste Korngrösse.* Backen- und Schneidezähne frisch geschlachteter Rinder wurden sorgfältig von Pulpa und anhängenden Zahnfleisch- und Knochenresten befreit und in einem grossen Stahlmörser zerstossen. Dabei musste darauf geachtet werden, dass keine Erwärmung des Zahnpulvers durch zu langes oder zu kräftiges Zerstossen eintrat. In flüssiger Luft vorgekühlte Zähne liessen sich zwar leichter zerstossen, zeigten aber in der Enzymaktivität keinen Unterschied. Das Zahnpulver wurde anschliessend durch einen Siebsatz in 5 verschiedene Korngrössen getrennt.

Je 2 g des Zahnpulvers wurden mit 10 cc destilliertem Wasser und einigen Tropfen Toluol 1½ Std. auf der Schüttelmaschine geschüttelt, danach abfiltriert und die Phosphatase-Aktivität nach der Dinatriumphosphat-Methode (DPP-Methode) gemessen. Ein Beispiel der Ergebnisse eines solchen Versuches, bei dem jeweils vom gleichen Ausgangsmaterial 2 Ansätze (Versuch A und B) gemacht wurden, zeigt Tabelle I.

TABELLE I

Korngrösse	Aktivität in mg-Einh./DPP	
	Versuch A	Versuch B
Nr. (1) 1 -1,5 mm	19	9
Nr. (2) 0,5 -1 mm	37,5	38,5
Nr. (3) 0,3 -0,5 mm	47,5	48,5
Nr. (4) 0,15-0,3 mm	34	47
Nr. (5) unter 0,15 mm	17	20,5
Nr. (6) 2 Std. in Kugelmühle gemahlen unter merk- licher Erwärmung; Korngrösse unter 0,15 mm	15	12

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Bei den Zähnen verschiedener Tiere ergab sich nicht immer das gleiche Aufteilungsverhältnis. In allen Versuchen aber zeigte die aus der Korngrösse Nr. 3 gewonnene Enzymlösung die höchste Phosphataseaktivität. Bei gleichem Ausgangsmaterial liessen sich die Werte mit einer Genauigkeit von \pm einer mg-Einheit der Dinatriumphosphat-Methode (DPP) reproduzieren.

(2) *Einfluss der Extraktionsdauer.* Der Einfluss der Schüttelzeit auf die Aktivität des Extraktes ergab die in Tabelle 2 verzeichneten Werte. (5 verschiedene Versuche, A-E).

TABELLE 2

Schüttelzeit bei Zimmertemperatur	Aktivitäten in mg-Einheiten/DPP				
	Vers. A	B	C	D	E
0,5 Std.	25	—	—	—	—
1 Std.	27	29,5	27,5	—	37,5
1,5 Std.	37,5	39	35,5	38	47
2 Std.	34	37	28	—	46,2
2,5 Std.	26,5	—	—	—	—
3 Std.	26	26	25	28	—
3,5 Std.	22	—	—	—	—
4 Std.	20	—	21	—	—
2 + 24 ^h Stehen	—	—	—	—	36,1
3 + 24 ^h Stehen ohne Schütteln	—	—	—	31	—
24 Std. Stehen	—	—	—	28,5	38,5

Das Verhältnis änderte sich auch hier etwas mit verschiedenem Ausgangsmaterial. Die beste Aktivität hatte immer ein etwa 1½ bis 2 Std. geschüttelter Extrakt.

(3) *Einfluss der Wassermenge bei der Extraktion.* Als dritter für die Extraktion wichtiger Faktor wurde das optimale Verhältnis von Zahnpulver zur Wassermenge beim Extrahieren ermittelt.

TABELLE 3

25 g Zahnpulver + g Wasser	Verhältnis	Aktivitäten in mg-Einheiten/DPP Schüttelzeit	
		3 Std.	2 Std.
100	1 : 4	41,5	46,5
125	1 : 5	33,0	40,5
187,5	1 : 7,5	33,5	38,5
250	1 : 10	17,0	29,0
375	1 : 15	11,5	—

Das Verhältnis 1:4 ist also am günstigsten.

(4) *In Lösung-Bringen von anorganischem Material.* Auflösen des anorganischen Materials in Säure bedingt selbstverständlich eine Inaktivierung des Enzyms. Ob ein schonendes Lösen unter Anwendung von Komplexbildnern vorteilhaft wäre, sollte geprüft werden. Zu diesem Zweck wurde mit einer 1 %igen Trilon-B-Lösung (Aethylendiamintetraessigsäure) in Verhältnis 1:10 Teilen Lösung 2 Std. lang geschüttelt. Das Trilon musste anschliessend durch 3-tägige Dialyse gegen fliessendes Leitungswasser quantitativ wieder entfernt werden, weil es die Farbreaktion des Phosphatase-Nachweises stört. Tabelle 4 zeigt das Ergebnis.

TABELLE 4

Extraktionslösung	mg-E/DPP
dest. Wasser mit Veronal-Puffer neutralisiert	34,0
1% Trilon-B-Lösung pH 4,1	24,8
desgleichen pH 7,0 (mit Veronal-Puffer neutralisiert).	18,4

Die Anwendung von Trilon bringt also keinen Vorteil. Möglicherweise ist die geringere Aktivität hier nur durch die Dialyse bedingt, auf die aber aus den oben genannten Gründen nicht verzichtet werden konnte.

(5) *Dialyse und Reaktivierung.* Von einer normal mit destilliertem Wasser extrahierten Enzymlösung wurde ein Teil erst 9 Tage gegen fliessendes Leitungswasser bei Zimmertemperatur, danach 3 Tage gegen öfter erneuertes destilliertes Wasser im Eisschrank bei +2 bis 4° dialysiert.

Der Rest blieb unter Toluol als Kontrolllösung unter gleichen Temperaturbedingungen aufbewahrt.

Nach Ablauf der 12 Tage wurde eine Probe des Dialysats mit DPP-Substratlösung ohne Mg^{2+} , eine zweite in Gegenwart von Mg^{2+} (1×10^{-3} m) und eine dritte Probe nach Zusatz der gleichen Menge einer frischen, durch Hitze völlig inaktivierten Enzymlösung gemessen. In den beiden letzten Fällen zeigte sich eine zwar deutlich erkennbare, aber nur geringe Reaktivierung, wie Tabelle 5 zeigt.

TABELLE 5

Kontrolllösung	mg-E/DPP
Gleich nach Extraktion	47
Nach 9 Tagen bei 23° + 3 Tagen bei 2-4°	33,5
Nach 9 Tagen bei 23° + 24 Tagen bei 2-4°	25
Dialysat	
Nach 9 Tagen bei 23° + 3 Tagen bei 2-4°	4,5
Nach 9 Tagen bei 23° + 3 Tagen bei 2-4° als Mischung 1:1 mit inakt. Enzymlösung	7,0
Nach 9 Tagen bei 23° + 3 Tagen bei 2-4° mit Zusatz von Mg^{2+} 1×10^{-1} m	5,6

(6) *Endgültige Versuchsanordnung.* Auf Grund der geschilderten Vorversuche wurde für den Vergleich der verschiedenen Methoden die Gewinnung der Ferment-extrakte folgendermassen vorgenommen.

Die Zähne wurden in einem Stahlmörser vorsichtig auf eine Korngrösse von 0,3 bis 0,5 mm zerkleinert, von Stücken kleinerer bzw. grösserer Korngrösse wurde durch Absieben getrennt. Das gemörserte Gut wurde mit der vierfachen Menge destillierten Wassers unter Zusatz von einigen Tropfen Toluol 1,5 Stunden lang geschüttelt. 1 bzw. 2 cc der filtrierten Lösung (vgl. Tabelle 6) wurden als Enzymlösung für die Bestimmung der Phosphatase-Aktivität nach den verschiedenen Methoden verwandt. Die Inkubationsdauer schwankte je nach der angewandten Methode (vgl. Table 6) zwischen 15 und 60 Minuten. Als Puffer fand ein Carbonat-Puffer Verwendung, der nach KING und DELORY (1945) die Phosphatase-Bestimmung nicht stört.

II. Methoden für den Nachweis der Phosphatase-Aktivität

Zunächst erfolgt nur eine kurze Schilderung der bekannten, für die vorliegenden Zwecke lediglich modifizierten Methoden. Ausführlich wird dann die neue Methode geschildert. Die mit den verschiedenen Methoden erzielten Ergebnisse werden am Schluss im Zusammenhang dargestellt.

(1) *Bestimmung mit dem Substrat Dinatrium- β -Glycerophosphat (β -GP, Tabelle 6).* Die von der Phosphatase aus dem Substrat abgespaltene Menge Phosphorsäure ist hier das Mass für die Aktivität des Enzyms. Unter Auswertung zahlreicher Vorschläge und Anregungen, besonders auf den Arbeiten von O. BODANSKY (1957), A. BODANSKY (1957), SHINOWARA, JONES und REINHART (1942) sowie THEORELL (1931) basierend, wurde die Methode so empfindlich gestaltet, dass sie zur Bestimmung der Phosphatase in Zähnen geeignet war.

Die optimalen Hydrolyse-Bedingungen für dieses Substrat wurden bei einer Hydrolysen-Dauer von 60 Minuten bei 37,5° bei dem pH-Wert 8,9 und der Substrat-Konzentration 5×10^{-3} m ermittelt. Hydrolyse und Abhängigkeit der Aktivität von der Enzym-Konzentration waren bis zu einer Stunde linear.

In der Literatur finden sich unterschiedliche Definitionen für eine Phosphatase-Einheit. Nach BODANSKY (1933) ist es die Ferment-Menge in 100 cc Serum, die in einer Stunde bei 37° beim pH 8,6 aus β -Glycerophosphat 1 mg Phosphor in Freiheit setzt. Nach ROCHE und BOUCHILLOUX (1950) ist es die Phosphatase-Menge, die in einer Minute bei 37° beim pH 9,9 aus β -Glycerophosphat 1 γ Phosphor abspaltet. Eine Phosphatase-Einheit nach SCHMIDT und THANNHAUSER (1943) ist die Ferment-Menge, die in 15 Minuten beim pH 9,3, aktiviert mit $MgCl_2$, 0,1 mg Phosphor abspaltet.

(2) *Bestimmung mit dem Substrat Dinatriumphosphat (DPP, Tabelle 6).* Bei dieser von KING und ARMSTRONG (1934) entwickelten Methode wird das durch die Phosphatase abgespaltene Phenol nach FOLIN und CIOCALTEU (1927) gemessen. GOMORI (1949, 1954) bestimmt das Phenol mit einem Diazo-Reagenz, KING und DELORY (1939) und RUPPERT (1950) messen das abgespaltene Phosphat. Für unsere Versuche werteten wir Anregungen von FOLLEY und KAY (1935), BUCH und BUCH (1939), LINHARDT und WALTER (1951) und KIRBERGER und MARTINI (1950) aus, um die Aktivität der Zahnphosphatase besser erfassen zu können. Für die enzyma-

tische Hydrolyse des Substrates Dinatriumphenylphosphat bei 37,5° wurde das pH-Optimum 10,2 bei der optimalen Substrat-Konzentration 1×10^{-2} m gefunden. Bei optimalen Bedingungen-Aktivierung mit Magnesiumsulfat (1×10^{-3} m)- verlief die Hydrolyse bei 37,5° nur bei einer Inkubationsdauer bis zu 30 Minuten.

In der Literatur wird bei dieser Methode eine Phosphatase-Einheit definiert als diejenige Enzym-Menge in 100 cc Serum, die in 30 Minuten bei 37° beim pH 9,3 und der Substrat-Konzentration von 5×10^{-3} molar aus Dinatriumphenylphosphat 1 mg Phenol freimacht (KING und ARMSTRONG, 1934). Nach KIRBERGER und MARTINI ist es die Enzym-Menge in 100 cc Blut, die in 15 Minuten bei 37° beim pH 9,9 und der Substrat-Konzentration von 1×10^{-2} m 1 mg Phenol abspaltet.

(3) *Bestimmung mit dem Substrat Tetracolaminphenolphthaleinphosphat* (CPP, Tabelle 6). Die von HUGGINS und TALALAY (1945) angegebene Methode bietet den Vorteil, dass das in Freiheit gesetzte Phenolphthalein bei alkalischer Reaktion direkt an seiner Eigenfarbe gemessen werden kann. Als Substrat wurden verwandt: Von HUGGINS und TALALAY sowie von JANECKE und DIEMAIR (1949) das Natriumphenolphthaleinphosphat, von STIVEN (1947) das Calciumphenolphthaleinphosphat, von LINHARDT und WALTER (1951) die Pyridin-Komplexverbindung der Phenolphthaleinphosphorsäure und von MATTENHEIMER (1953) das in der Zeiteinheit etwas stärker gespaltene Tetracolaminphenolphthaleinphosphat.

Bei dieser durch ihre Einfachheit ausgezeichneten Methode wird bei Versuchsende ein "Farbreaktions-Puffer" zugegeben, der durch seine hohe Alkalität und seinen Gehalt an Pyrophosphat die Wirkung des Enzyms unterbricht und gleichzeitig den pH-Wert der Messlösung auf 10,6–10,9 verschiebt. Hier besitzt das (abgespaltene) Phenolphthalein bei hoher Farbintensität eine ausreichende Farbbeständigkeit (1 Stunde). Die Methode bietet auch die Möglichkeit, den Verlauf der hydrolytischen Spaltung an der zunehmenden Farbtiefe während der Fermenteinwirkung direkt zu verfolgen, wobei allerdings, da man den die Spaltung unterbrechenden Farbreaktionspuffer nicht zugibt, die Farbintensität nicht voll entwickelt ist. Mit dem Substrat Tetracolaminphenolphthaleinphosphat wurde für die Zahn-Phosphatase das pH-Optimum 9,7/37,5° bei der optimalen Substrat-Konzentration 1×10^{-3} m gefunden. Der Verlauf der Hydrolyse erwies sich bis zu 60 Minuten streng linear, wenn die jeweils abgespaltene Phenolphthalein-Menge in Einheiten umgerechnet wurde: 2 cc Enzym-lösung (–0,5 g Zahn) spalteten bei 37,5° unter optimalen Bedingungen ab:

Hydrolyse-Zeit in Minuten	abgespaltenes Phenolphthalein	Einheiten, auf die jeweilige Spaltungs- zeit bezogen	Verhältnis
15	4 γ	3,6	1
30	10,5 γ	6,2	2
60	28 γ	12,4	4

Ebenso zeigten die abgespaltenen Mengen Phenolphthalein im doppelt logarithmischen Koordinaten-System eine streng lineare Abhängigkeit zur Enzym-Konzentration bis zu Inkubations-Zeiten von 2 Stunden (Abb. 1).

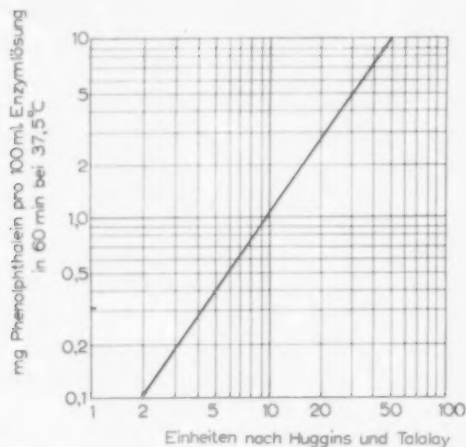


ABB. 1. Eichkurve zur Ablese der Phosphatase. Einheiten nach HUGGINS und TALALAY aus der abgespaltenen Menge Phenolphthalein.

(4) *Bestimmung mit dem Substrat Dinatrium-p-nitrophenylphosphat (PNP, Tabelle 6).* Dinatrium-p-nitrophenylphosphat wurde nach OHMORI (1937) durch Nitrierung von Dinatrium-phenylphosphat dargestellt. Die Messung der Farbe des abgespaltenen p-Nitrophenol in alkalischem Milieu bei 400 m μ lässt die Fermentwirkung unmittelbar deutlich werden.

Aus den Anregungen der von den Autoren OHMORI (1937), BESSEY, LOWRY und BROCK (1946) und ANDERSCH und SZCZYPINSKY (1947) beschriebenen Methoden wurde zur Bestimmung der Phosphatase in Zähnen ein besonderer Ansatz ausgearbeitet. Die Art des Puffers ist nach MORIMOTO (1937) ohne Einfluss auf die Farbintensität des p-Nitrophenols. Nach BESSEY *et al.* soll die aktivierende Wirkung von Magnesium bei diesem Substrat wesentlich geringer sein als bei β -Glycerophosphat. Ein scharfes pH-Optimum wird beim pH-Wert, 10,0–10,1 angegeben.

Die optimalen Hydrolyse-Bedingungen wurden von uns bei dem pH-Wert 9,9/37,5° und der Substrat-Konzentration 5×10^{-3} m gefunden. Durch Magnesiumsulfat (1×10^{-3} m) liess sich eine etwa 20 %ige Aktivierung erzielen. Die abgespaltenen Mengen p-Nitrophenol waren bis zu 40 Minuten den Hydrolyse-Zeiten proportional.

Eine Phosphatase-Einheit nach BESSEY u.a. ist die Enzym-Menge in 100 cc Serum, die in 60 Minuten bei 38° beim pH 10 und der Substrat-Konzentration von 6×10^{-3} m 1 mMol p-Nitrophenol abspaltet.

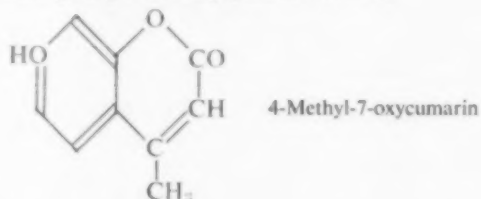
(5) *Bestimmung mit dem Substrat o-Carboxyl-phenylphosphorsäure (OCP, Tabelle 6).* Die von BRANDENBERGER und HANSON (1953) sowie von HOFSTEE (1954) angegebene Methode beruht darauf, dass das Substrat bei 295–310 m μ eine sehr geringe, die von Phosphatasen leicht abgespaltene Salicylsäure in diesem Bereich dagegen eine sehr starke Absorption besitzt. Die optimalen Hydrolyse-Bedingungen wurden von uns bei der Substrat-Konzentration 1×10^{-2} m und dem pH 9,8/37,5° und einer Mg²⁺-

Konzentration von 1×10^{-3} m ermittelt. Eine lineare Abhängigkeit zwischen Enzym-Konzentration und abgespaltener Salicylsäure-Menge bestand bei $37,5^\circ$ nur bei Spaltungszeiten bis zu 30 Minuten.

Eine Einheits-Definition wurde in der Literatur für diese Methode nicht beschrieben. Als Mass für die Aktivität der Phosphatase in einem Organ dienten HOFSTEE die von 1 mg Organsubstanz in 30 Minuten bei 30° beim optimalen pH-Wert aus dem Substrat ($3,3 \times 10^{-3}$ m) abgespaltenen Mikromole Salicylsäure.

(6) *Bestimmung mit dem Substrat β -Methylumbelliferonphosphorsäure* (MCP., Tabelle 6). NEUMANN (1948) berichtete über eine Möglichkeit zur Darstellung der Phosphorsäureester des Fluoreszeins, Eosins und β -Methylumbelliferons (4-Methyl-7-oxycumarin) und diskutierte die Möglichkeit ihrer Verwendung als Substrat für einen Phosphatase-Nachweis im histologischen Schnitt unter dem Fluoreszenz-Mikroskop. Irgendwelche experimentellen Angaben über die Ausarbeitung oder Anwendung einer solchen Methode zur Phosphatase-Bestimmung konnten jedoch in der Literatur nicht gefunden werden.

Dieser Hinweis lenkte jedoch die schon länger bestehende Absicht, eine Fluoreszenz-Methode zur quantitativen Phosphatase-Bestimmung auszuarbeiten, in die von NEUMANN angedeutete Richtung. Auf diese Weise konnte eine bisher nicht beschriebene Fluoreszenz-Methode zur quantitativen Phosphatase-Bestimmung ausgearbeitet werden, für die als Substrat der Phosphorsäureester des 4-Methyl-7-oxycumarins (= β -Methylumbelliferonphosphorsäure) gewählt wurde.



Die Darstellung des Phosphorsäureesters erfolgte durch Einwirkung von POCl_3 auf das in Pyridin gelöste Methyloxycumarin, anschliessender Hydrolyse mit Wasser und Ausfällung des Esters mit Salzsäure. Der Ester, ein weisses, feinkristallines Pulver, ist im Gegensatz zum Methyloxycumarin leicht wasserlöslich und zeigt im alkalischen Gebiet eine kaum merkliche Fluoreszenz. Da die starke Fluoreszenz des Methyloxycumarins im UV nur im alkalischen Gebiet über pH 8,0 existiert, kann mit diesem Substrat an der Zunahme der Fluoreszenz nur die Hydrolysewirkung einer alkalischen Phosphatase direkt und kontinuierlich beobachtet werden. Zur Bestimmung wurde folgendermassen vorgegangen.

3 cc Pufferlösung (0,2 m) pH 9,4/ $37,5^\circ$ wurden mit 1 cc Substratlösung (3×10^{-3} m), enthaltend Magnesiumsulfat (6×10^{-3} m) als Aktivator, versetzt, einige Minuten auf $37,5^\circ$ vorgewärmt, 2 cc Enzymlösung zugegeben und dann inkubiert.

Hierbei konnte der Verlauf der enzymatischen Esterspaltung an der Zunahme der Fluoreszenz, auch unter beliebig gewählten Bedingungen (Temperatur, pH-Wert, Substrat-Konzentration- Enzym-Konzentration usw.) zeitlich kontinuierlich verfolgt werden. Die Anregung der Fluoreszenz erfolgte durch monochromatisches UV-Licht

(366 m μ). Hinter der Küvette, vor der lichtempfindlichen Zelle, befand sich ein zweites Filter, welches nur die emittierte Fluoreszenzstrahlung, nicht aber die anregende UV-Strahlung durchliess. Die Ablesung der Fluoreszenz-Intensität erfolgte auf der üblichen in % D bzw. Extinktionswerte eingeteilten Skala, wie sie für die Absorptionsmessungen verwendet wird.

Da bei der Fluoreszenzmessung aber die Verhältnisse umgekehrt als bei Absorptionsmessungen liegen, kann eine geradlinige Eichkurve nicht ohne weiteres durch Auftragen der abgelesenen Extinktionen erhalten werden. Niedrigen Konzentrationen, d.h. geringer Fluoreszenz, entsprechen hohe "Extinktionswerte"—starker Fluoreszenz entsprechen niedrige "Extinktionswerte". Eine so erhaltene Eichkurve zeigt etwa die Form einer Hyperbel.

Zu einer annähernden Geraden gelangt man, wenn man (1) die Skala umdreht, d.h. den gemessenen Durchlasswert von der Zahl 100 subtrahiert ($100-D$) und dann den diesem Durchlasswert (D') entsprechenden Extinktionswert (E') aufträgt und (2) mit der höchsten gewählten Methylnumbelliferon-Konzentration als Standard-Fluoreszenz (z.B. 1 μ Mol (6 cc) die Skalenanzeige willkürlich und für jede weitere Messung auf 100 % D einstellt (Abb. 2).

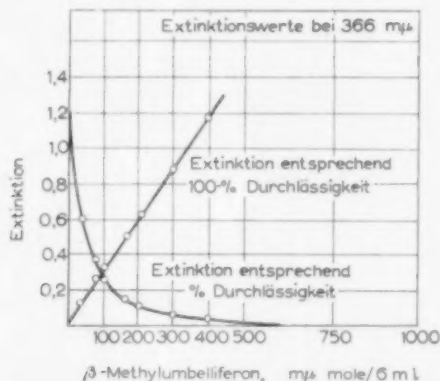


Abb. 2. Eichkurven zur fluoreszenzanalytischen Ermittlung des abgespaltenen β -Methylumbelliferons aus den Extinktionswerten (E) und aus den umgewandelten Extinktionswerten (E') bei 366 m μ für die Phosphatase-Bestimmung mit β -Methylumbelliferonphosphorsäure.

Ein fast genau linearer Verlauf wurde erhalten, als die E' -Werte gegen die Konzentrationen in einem doppelt logarithmischen Koordinaten-System eingetragen wurden.

Ein Vergleich der mit den verschiedenen Methoden erzielten Werte ist nur möglich, wenn man eine vergleichbare Definition der Fermenteinheiten verwendet. Wie unterschiedlich dies bei den verschiedenen Methoden bisher erfolgt ist, dürfte aus den bei diesen gemachten Ausführungen deutlich geworden sein. Es wird deshalb vorgeschlagen, die folgenden zwei Definitionen der Phosphatase-Einheit zu verwenden:

(a) Eine mg-Einheit ist diejenige Phosphatase-Menge in 100 cc Enzymlösung, die bei optimaler Substrat-Konzentration und beim pH-Optimum, aktiviert mit Mg^{2+} , bei 37,5° in 60 Minuten 1 mg eine der Esterkomponenten des Substrates abspaltet.

(b) Eine $\mu\text{Mol-Einheit}$ ist diejenige Phosphatase-Menge in 1 g der zu untersuchenden Organ-Substanz, die analog (a) unter optimalen Bedingungen bei $37,5^\circ$ in 60 Minuten 1 μMol eine der Phosphorsäureesterkomponenten des Substrates abspaltet.

In jeder Angabe eines Messergebnisses in einer dieser Phosphatase-Einheiten müssen dann aber noch alle speziellen Massbedingungen enthalten sein, die in den Grunddefinitionen nicht festgelegt sind: (1) Name des verwendeten Substrates, (2) genaue Definition der Enzymlösung, enthaltend Zerkleinerungsgrad der zu untersuchenden Organ-Substanz, Mengenverhältnis der Organ-Substanz zum Extraktionsmittel, Schütteldauer der Extraktion und Schütteltemperatur, (3) Art und Konzentration der Pufferlösung, (4) Konzentration des aktivierenden Magnesiumsalzes.

Die Fermenteinwirkungs-dauer zur Bestimmung der Phosphatase-Einheit ist in der Grunddefinition auf 60 Minuten festgelegt. Es ist aber zu beachten, dass nicht bei allen Methoden der tatsächlich nach 60 Minuten abgelesene Wert genommen werden kann. Der nach 30 Minuten gemessene und dann mit 2 multiplizierte Wert ist nur dann richtig, wenn die abgespaltene Menge zur Spaltungszeit bis zu 60 Minuten in geradlinigem Verhältnis steht. Bei den meisten Bestimmungs-Methoden sinkt aber die Anfangsgeschwindigkeit der Hydrolyse nach 15–30 Minuten schon merklich ab. Man erhält daher nach 60 Minuten zu niedrige Werte. In diesen Fällen ist für die zur Bestimmung der Phosphatase-Einheit definierte Einwirkungszeit nur die Dauer zu wählen, bis zu welcher die zeitliche Abspaltung linear verläuft, d.h. wo noch die Anfangsgeschwindigkeit der Hydrolyse vorherrscht. Die Vergleichsmöglichkeit der Einheiten bleibt dadurch erhalten, dass die definitionsgemäss pro Einheit abgespaltene Menge im gleichen Verhältnis zur verkürzten Fermenteinwirkungs-dauer herabgesetzt wird.

Für eine Phosphatase-Einheit wird also die Abspaltung festgelegt: In der Grunddefinition 1 mg (bzw. 1 μMol) in 60 Minuten, in einem anderen Falle z.B. 0,5 mg (bzw. 0,5 μMol) in 30 Minuten denn nach 60 Minuten Einwirkungs-dauer würde im zweiten Falle nicht 1 mg abgespalten, sondern aus oben angeführten Gründen weniger.

Als Anwendungsbeispiel sei das der ausführlich beschriebenen Fluoreszenz-Methode gegeben. Bei Anwendung dieser Methode werden die Einheiten folgendermassen definiert:

Eine $\mu\text{Mol-Einheit}$ ist die Phosphatase-Menge in 1 g Zahn, die aus β -Methylumbelliferonphosphorsäure in einer Substrat-Konzentration von 5×10^{-4} m beim pH-Optimum 9,4, aktiviert mit Magnesiumsulfat (1×10^{-3} m), bei $37,5^\circ$ in 15 Minuten 0,25 μMol β -Methylumbelliferon abspaltet.

Eine mg-Einheit ist die Phosphatase-Menge in 100 cc Enzym-Lösung aus 25 g Zahnpulver der Korngrösse 0,3–0,5 mm (1,5 Std. geschüttelt), die unter den gleichen Bedingungen in 15 Minuten 0,25 mg β -Methylumbelliferon abspaltet.

III. Vergleich der mit verschiedenen Methoden erhaltenen Werte für die Phosphatase-Aktivität

In Tabelle 6 sind die Ergebnisse zusammengefasst, die erhalten wurden, wenn die gleiche Enzymlösung mit allen Methoden hintereinander gemessen wurde. Eine

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derartige Gesamtmessung ist 4-mal, jeweils durch Wochen getrennt, stets mit frischen Lösungen, in Doppelbestimmungen durchgeführt worden. Die mit den einzelnen Methoden gefundenen Einheiten standen stets untereinander im gleichen Verhältnis. (Die Ergebnisse sind in Tabelle 6 zusammengestellt.)

TABELLE 6

Substrat	PNP	DPP	MCP	β -GP	CPP	OCP
Substrat-Konzentration	5×10^{-3} m	1×10^{-3} m	5×10^{-4} m	5×10^{-3} m	1×10^{-3} m	1×10^{-3} m
pH/37,5°	9,9	10,2	9,4	8,9	9,7	9,8
Inkubat. Zt. in Min./37,5°	15	15	15	60	60	30
cc Enzymlösung (aktiviert mit 1×10^{-3} m Mg^{2+})	1	1	2	2	2	2
Extinktion	1,699	1,510	0,801	0,900	0,863	0,427
1 μ Mol=	139,1 γ <i>p</i> -Nitro-phenol	94,1 γ Phenol	176,1 γ Methyl-oxy-cumarin	98 γ Phosphor-säure 31 γ Phosphor	318,3 γ Phenol-phthalein	138 γ Salicyl-säure
abgespaltene γ	73,7	102	46,7	41,5	70	96,6
abgespaltene μ Mole	0,530	1,084	0,265	1,339	0,220	0,700
mg-Einheiten	29,5	40,8	9,3	2,07	24,5*	9,66
μ Mol-Einheiten	8,48	17,34	2,12	2,68	—	2,80
* Einheiten nach HUGGINS und TALALAY						

IV. Bestimmung der alkalischen Phosphatase in reinem Schmelz und Dentin

Die grundsätzliche Frage nach der Existenz einer extrahierbaren alkalischen Phosphatase im Säugerzahn war durch unsere Versuche in positivem Sinne beantwortet worden. Dass die Hauptmenge des Ferments im Dentin vorlag, konnte vermutet werden. Da WHITE und HESS in ihren gründlichen Untersuchungen eine extrahierbare Phosphatase in Dentin nicht hatten nachweisen können, sollte ein abschliessender Versuch darüber Klarheit bringen. Zum Nachweis wurden die Methoden verwandt, die sich bei uns als besonders empfindlich erwiesen hatten (PNP und DPP. vgl. Tabelle 6). Weiterhin wurde versucht, ob ein Nachweis extrahierter Phosphatase im Schmelz möglich war. Zu diesem Zweck wurde bei einigen Rinderschneidezähnen der Schmelz vom Dentin mechanisch durch Absprengen getrennt und von anhaftenden Dentinresten durch Ausbohren und Abschleifen vorsichtig so befreit, dass eine Erwärmung der Zahnschmelzsubstanz weitmöglich vermieden wurde. Es wurde nur die labiale Seite verwendet, da die palatinale Seite eine zu dünne Schmelzschicht besitzt. Der so erhaltene reine Schmelz wurde in einem Stahlmörser vorsichtig auf eine Korngrösse von 0,3–0,5 mm zerdrückt und mit der 4-fachen Menge

destilliertem Wasser sowie einigen Tropfen Toluol 1,5 Stunden lang geschüttelt. Auch wenn die Inkubationszeit auf das 4-fache—60 Minuten—ausgedehnt wurde, war eine Fermentaktivität im Schmelz nicht nachzuweisen.

Zur Bestimmung der Phosphataseaktivität im Dentin wurde in gleicher Weise vorgegangen wie im Abschnitt I/6 beschrieben. Mit der DPP-Methode wurde eine Aktivität von im Mittel 48,5 mg-Einheiten gefunden. Dieser Wert passt gut zu dem in Gesamt-Zahnschmelz erhaltenen (40,8, vgl. Tabelle 6). Der mit reinem Dentin erhaltene etwas höhere Wert entspricht der Erwartung, da bei Verwendung von Gesamt-Zahnschmelz hier auch der inaktive Schmelz enthalten war.

V. Diskussion der Ergebnisse

Die zur Bestimmung der Phosphatase in Organen und Körperflüssigkeiten angewandten Methoden weichen z.T. recht weit von einander ab, die erzielten Ergebnisse sind daher nicht ohne weiteres vergleichbar. Neben der Frage eines Vorkommens einer extrahierbaren Phosphatase im Zahn waren daher ein Vergleich der verschiedenen Methoden und die Aufstellung vergleichbarer Einheiten ein wesentliches Anliegen der Arbeit. Grundsätzlich sind die beschriebenen 5 Standard-Methoden alle brauchbar, doch weist ihre Empfindlichkeit beträchtliche Unterschiede auf. Gemessen an Inkubationszeit, Enzym-Menge und Extinktionswerten sind PNP und DPP etwa 15-fach empfindlicher als CPP, die aber wiederum durch ihre Einfachheit besticht. Die neu beschriebene Methode (MCP), von der wir uns als Fluoreszenzmethode besonders viel versprochen, hat bisher nur etwa 25 % der Empfindlichkeit der beiden obengenannten Methoden. Es steht jedoch zu erwarten, dass hier durch bessere Fluoreszenzmessung eine erhebliche Steigerung möglich wird. Dies sollen weitere Versuche ergeben.

Ein Vergleich der von uns gefundenen Werte der Phosphataseaktivität mit den von KREUDENSTEIN angegebenen ist in Anbetracht des verschiedenen methodischen Vorgehens kaum möglich. Doch dürfte interessant sein, die verschiedenen Aufarbeitungs-Methoden einmal mit gleichen Nachweismethoden zu vergleichen.

Die erheblichen Differenzen unserer Ergebnisse zu den von WHITE und HESS angegebenen mögen z.T. daran liegen, dass WHITE und HESS an menschlichen Zähnen, wir an Rinderzähnen arbeiteten. Eine andere Erklärungsmöglichkeit liegt darin, dass die bei energischer Zerkleinerung der Zahnschmelz in der Wiley-Mühle sich entwickelnde Hitze das Ferment zerstört hat. Wie hitzeempfindlich dieses ist, ergibt sich aus unseren Untersuchungen über Korngrösse und Vermahlungszeit (vgl. Abschnitt I/1 und Tabelle 1) sowie daraus, dass Erhitzen der extrahierten Fermentlösung zu schneller Inaktivierung führte. Bei der Zerkleinerung von Knochen führt das gleiche Verfahren vermutlich deshalb nicht zu einer Inaktivierung gleichen Ausmasses, weil die weniger grosse Härte der Knochensubstanz bei der Zerkleinerung nicht zu so starker Erwärmung führt, wie es bei Zahnschmelz der Fall ist. Ein Vergleich der von uns mit der von WHITE und HESS erzielten Fermentaktivität zeigt, dass unter Berücksichtigung von Substrat-Menge und Inkubations-Zeit die von uns im Extrakt gemessene Aktivität etwa um das 20-fache höher liegt. Die von WHITE und HESS gefundene Substrat-gebundene Aktivität ist daher möglicherweise nur eine

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geringe Restaktivität, die auch bei der beim Zerkleinern auftretenden Erhitzung noch erhalten blieb. Nach unseren Untersuchungen scheint an der Existenz einer extrahierbaren Phosphatase im Rinderzahn nicht zu zweifeln zu sein.

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RADIOAUTOGRAPHIC INVESTIGATION OF THE UPTAKE OF LABELLED METHIONINES BY THE DENTINE AND ENAMEL MATRIX OF GROWING TEETH

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Abstract—Radioautography after injection of labelled methionine into young rats and adult mice yielded the same distribution in growing teeth whether the label was S^{35} , C^{14} or H^3 . Assuming that radioactivity uptake indicates protein formation, it was concluded that proteins of dentine and enamel matrix arise as follows. In the dentinal organ, a protein presumed to be collagen or a collagen precursor appears within the cytoplasm of odontoblasts and is then released into dentinal collagen. In the enamel organ, a protein appears in the cytoplasm of ameloblasts and is then released into pre-enamel, eventually forming the enamel matrix.

INTRODUCTION

It is believed that sites of protein synthesis may be detected radioautographically in growing teeth after injection of various protein precursors, such as bicarbonate- C^{14} (GREULICH and LEBLOND, 1953), glucose- C^{14} (KUMAMOTO and LEBLOND, 1958) and particularly labelled amino acids (LEBLOND, EVERETT and SIMMONS, 1957). Thus, a remarkably high uptake of radioactivity was observed in pre-enamel after injection of labelled methionine (BÉLANGER, 1956) and in predentine after injection of labelled glycine (CARNEIRO and LEBLOND, 1959). It was decided to reinvestigate the entry of methionine into pre-enamel for the following reasons. Since the radioautographic image over pre-enamel after injection of methionine- S^{35} (BÉLANGER, 1956) was somewhat similar to that obtained with sulphate- S^{35} (BÉLANGER, 1955), it was possible that the isotopic sulphur had been broken off from the amino acid and had become incorporated by the tissues as sulphate rather than methionine. Therefore, it was proposed to examine the distribution of methionine labelled with tracers other than S^{35} . Both the methyl- C^{14} and methyl- H^3 isotopic forms of methionine were used, but the lower β -ray energy of H^3 allowed a better radioautographic resolution than either the sulphur or carbon-label.

MATERIALS AND METHODS

Four male littermate white mice, each weighing 10–14 g, were given a single subcutaneous injection of 10 μ c L-methionine methyl- C^{14} in 0.04 ml of saline per gram body weight. The animals were sacrificed 1 hr, 3 hr, 24 hr and 6 days later, following ether anaesthesia.

Ten male C_3H mice, weighing 26–34 g each, were injected, as above, using a dose of 5 μ c/g body weight of methionine-methyl- H^3 in 0.01 ml of saline. They were sacrificed in pairs 30 min, 4 hr, 35 hr, 7 days and 35 days later.

Twenty male C_3H mice, weighing 14–16 g each, were injected subcutaneously using 4 μ c DL-methionine- S^{35} in 0.0064 ml saline per gram body weight. They were sacrificed in pairs 1 hr, 4 hr, 35 hr, 4 days, 7 days, 21 days, 28 days, 35 days and 45 days later, after ether anaesthesia.

In another experiment, 20 male 3 day old black-and-white hooded rats weighing 7–10 g were employed. They were injected with 5 μ c DL-methionine- S^{35} in 0.01 ml saline per gram body weight and were sacrificed in pairs 30 min, 3 hr, 8 hr, 24 hr, 3 days, 6 days, 14 days, 30 days, 60 days and 90 days later.

The jaws were fixed in alcohol-formalin and decalcified in Versene. Some of the slides for radioautography were stained with haematoxylin-eosin; the rest were left unstained.

Slides to be radioautographed were dipped in two baths of 1% celloidin in alcohol-ether (1:1), were allowed to dry, and, in the dark, were covered with melted NTB3 Kodak emulsion according to the dipping technique (MESSIER and LEBLOND, 1957). Both the NTB2 Kodak and the Ilford Nuclear Research (gel) Emulsion were also used in the methionine-methyl- C^{14} and methionine- H^3 experiments. The slides were stored in dry, light-tight containers kept at 4°C. The exposure time was evaluated by development of test slides. Radioautographs of underexposed, optimal and overexposed tissue sections were obtained (exposure time 1–7 months). The latent images formed by β -radiation energy on emulsion were developed producing a silver grain blackening over those areas of tissue containing radioactivity. The preparations were then passed through alcohols, cedar oil, and Canada balsam, and were permanently mounted with cover slips.

RESULTS

It should be emphasized that all Figures except Fig. 5 (Plate 2) are unstained, so that all dark or grey areas indicate uptake of radioactivity.

Enamel tissues

Young rats. The distribution pattern of the S^{35} label after methionine- S^{35} injection was examined in the incisors and molars 30 min after injection. Two intensely radioactive areas were seen. One was within the cytoplasm of the ameloblasts close to the nucleus and a few microns below the apical surface (facing pre-enamel), that is, approximately in the Golgi zone. Since ameloblasts are located side by side, and thus make up a regular columnar epithelium, the silver granules over this zone are at the same level in all the cells, resulting in a band-like reaction along the cell row (Fig. 1A, Plate 1). A second reactive band, more intense than the first one, was found over pre-enamel in immediate contact with the apical surface of the ameloblasts (Fig. 1, horizontal arrow).

In the young rats sacrificed 3 hr after injection, the intracellular radioactivity had nearly disappeared, but the pre-enamel band had increased in intensity. It may be recalled that the level of free labelled methionine in blood is high at 30 min or 1 hr after injection, but falls to a low value by the third or fourth hour (TARVER, 1954; LEBLOND *et al.* 1957), so that radioautographic reactions seen at any time interval

result from the uptake of the amino acid during the first and second hour after injection. By 8 and 24 hr, the radioactive band of pre-enamel gradually expanded over "young" enamel, while next to the ameloblasts, layers of pre-enamel with little or no radioactivity appeared. The band was broader and slightly less intense than earlier. This trend was continued at 1, 3 and 6 day intervals as the material covered by the band successively became "young" and "maturing" enamel. By the fourteenth day, the fate of the band could not be followed, as most of the tissue consisted of fully formed enamel (which was lost during decalcification). A small amount of "maturing" enamel matrix retained after decalcification near the junction of crown and root was only moderately radioactive.

Adult mice. The early distribution of methionine- S^{35} , $-C^{14}$ and $-H^3$ in the growing incisor teeth was essentially the same as described in the young rat. Radioautographs at 30 min and 1 hr after injection showed radioactivity over the cells (Fig. 3, A, Plate 1) and over pre-enamel (Fig. 3, horizontal arrow). At later time intervals, the cellular reaction decreased and eventually disappeared, while the pre-enamel band became maximal by 3 or 4 hr and later broadened into a diffuse band seen over young and maturing enamel, as shown at the horizontal arrow in Fig. 2 (Plate 1). (In the erupted molars of adult mice, the enamel was lost by decalcification and could not be investigated.)

Dentinal tissues

Young rats. On giving methionine- S^{35} to these, examination of incisor and molar teeth showed radioactivity in the cytoplasm of the odontoblasts and at the edge of predentine at 30 min after injection, but the two resulting reaction bands were weak and usually not distinguishable (except when artificially separated as at the top left in Fig. 1). At 3 hr, the cellular reaction had decreased and the predentine band became distinct. By 8 hr, part of the band was over dentine proper. Later, the band was exclusively over dentine and its distance from the odontoblasts increased with time, although its distance from the dentino-enamel junction was not altered; nor was its intensity changed even weeks or months after injection (as shown in a stained preparation made 6 days after injection, Fig. 5, and in an unstained one at 2 months, Fig. 6, Plate 2).

Adult mice. The same observations were made with the incisors of adult mice injected with methionine- S^{35} , $-C^{14}$ or $-H^3$. Thus, with methionine- H^3 , a rather weak band was seen at 30 min over odontoblasts (Fig. 3, O) and predentine (Fig. 3, vertical arrow). The odontoblast reaction decreased soon, while the predentine band intensified and was later seen over dentine (Fig. 4, Plate 2).

The molars of adult mice showed no appreciable reaction over odontoblasts, predentine or dentine.

DISCUSSION

The uptake of the methionine label followed the same pattern in the incisors and molars of young rats and in the incisors of adult mice, that is to say, in all rapidly growing teeth. No incorporation of the methionine label was seen in the non-growing

molars of adult mice. Hence the uptake of labelled methionine in these experiments must have been associated with growth and matrix formation in both the enamel organ and dentinal tissues.

Various views have been held regarding the role of methionine in the body. Following the injection of large doses of this substance into young rats, DU VIGNEAUD, RACHELE and WHITE (1956) observed that its methyl group was labile, passing into choline and other substances, and even into carbon dioxide. They concluded that methionine was a methyl donor. From this data it would be expected that methionine, labelled in its methyl group with C^{14} or H^3 , would produce a different radioautographic picture from that obtained with sulphur-labelled methionine. However, no differences were seen in the radioautographic patterns obtained with the three types of methionine in the tooth (Figs. 1-4) or elsewhere (unpublished results). Perhaps the small, physiological doses of methionine which we administered were not subjected to demethylation to a significant degree. Demethylation would occur mainly when excessive doses of methionine are used, as in the experiments of DU VIGNEAUD *et al.*

What then is the fate of methionine? The possibility of a mere adsorption of the labelled amino acid on tissue components was unlikely, since the multiple steps of histological processing (fixation, dehydration, staining, etc.) would tend to extract the amino acid. Furthermore, adsorption on the matrix would not explain the persistence of the reactions, especially in the dentine (Fig. 6), long after the blood level had fallen. The possibility of exchange of radioactive for non-radioactive amino acids within protein molecules was considered. However, unpublished results with leucine- H^3 gave an almost identical distribution as with methionine- H^3 . It is felt that this fact does not support the exchange theory, since two entirely different amino acids would not be likely to undergo exchange in exactly the same place. Furthermore, if exchange were involved, one would expect reactions to appear throughout the matrices rather than near the cells and also in non-growing, as well as in growing, molars. Assuming that methionine was not taken up by exchange, the last possibility was that this amino acid, like leucine, is utilized for protein synthesis.

Much evidence has accumulated that methionine is taken up as such into newly formed proteins (TARVER, 1954; LEBLOND *et al.* 1957). According to these authors, sites of deposition of the label reveal where the synthesis of methionine-containing proteins takes place. It is indeed known that the matrices supporting tooth minerals contain proteins. In enamel there is a very insoluble, little-known fibrous protein, while in dentine the protein material is almost exclusively composed of collagen (STACK, 1955). Methionine- H^3 would thus be taken up in the course of the synthesis of these proteins. Our radioautographic observations would provide a visual picture of the formation of matrical proteins in growing dentine and enamel.

Dentinal tissues. The uptake of methionine was rather low and, thus at the early interval, the picture was somewhat hazy, but in areas where the odontoblasts were artificially separated from predentine, it was possible to see that weak bands were present in both odontoblasts and predentine (top of Figs. 1 and 3). The decrease in the odontoblast radioactivity between 30 min and 3 hr, that is at the time when

labelled methionine disappeared from the blood, and the concomitant increase in predentinal band intensity, led us to conclude that the material appearing in the cells is the precursor of the labelled component of predentine. This interpretation was strengthened by a similar, but far more evident sequence of pictures after injection of tritium-labelled glycine. In this case, the radioactive material observed in the odontoblasts at 30 min after injection was clearly the precursor of the radioactive material appearing in predentine at 4 hr (CARNEIRO and LEBLOND, 1959). Semi-quantitative densitometric estimates revealed that the intensity of the radioautographic reaction was about twenty times stronger with glycine than with methionine (CARNEIRO and LEBLOND, 1959), a fact in agreement with a content of glycine twenty-eight times greater than that of methionine in collagen (BOWES and KENTEN, 1948). It was concluded that, with both precursors, we had visualized the elaboration of a collagen or collagen precursor by the odontoblasts and its eventual deposition as predentinal collagen.

Incidentally, the predentinal band soon became a dentinal band. The material of the band appeared to be indefinitely stable, since no change was seen in the intensity of the reaction with time (Figs. 4-6)—an observation in line with the stability of collagen in general (NEUBERGER and SLACK, 1953).

Enamel tissues. The observations reported in the present paper allowed visualization of the formation of enamel protein. At the 30 min interval, reactions were observed in a Golgi-like zone of the ameloblasts and in pre-enamel (Figs. 1 and 3); but, with time, the cellular reaction promptly disappeared, while the pre-enamel reaction intensified. This was taken to mean that a protein elaborated in a Golgi-like zone of the cell was promptly released outside the cell, thus providing material for building pre-enamel. Whether the newly formed protein made up the Tomes' processes or intermediate material, or probably both, could not be established. Later, the matrical reaction band became broader and less intense (Fig. 2), as the labelled region successively became "young", "maturing" and fully mature enamel. The increased diffuseness of the reaction was attributed to "dilution" of matrical material, due to the considerable addition of minerals taking place during enamel maturation.

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FIGS. 1-4. Coated radioautographs of unstained incisors taken from rats and mice at early (Figs. 1 and 3) or late (Figs. 2 and 4) intervals after radio-methionine injection. In Figs. 1-3, the sections are perpendicular to the axis of the incisor tooth; in Fig. 4, the cut is oblique. $\times 50$.

A, ameloblasts; O, odontoblasts; AB, alveolar bone; P, pulp; PM, periodontal membrane. The radioautographic reactions over the pre-enamel and enamel matrix are indicated by horizontal arrows, and over the predentine and dentinal matrix by vertical arrows.

FIG. 1. Three day old rat sacrificed 0.5 hr after methionine- S^{35} injection. (Five month exposure).

In the enamel organ, reaction bands may be seen over the ameloblasts (A) and pre-enamel (horizontal arrow). In the dentinal organ, faint bands may be distinguished over odontoblasts (O) and predentine (vertical arrow).

In alveolar bone, the isotope may be seen over osteoid matrix and adjacent bone (AB). Pulp (P) and periodontal membrane (PM) are slightly radioactive.

FIG. 2. Adult mouse sacrificed 6 days after methionine- C^{14} injection. (One month exposure).

In the enamel organ, radioactivity spreads out over a broad area, which corresponds approximately to the zones of young and maturing enamel. In the dentinal organ, a reaction band is observed over dentine. Other structures have lost most of the radioactivity which they contain soon after injection.

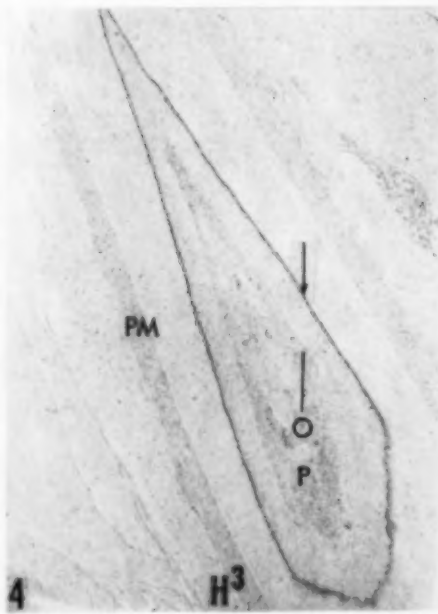
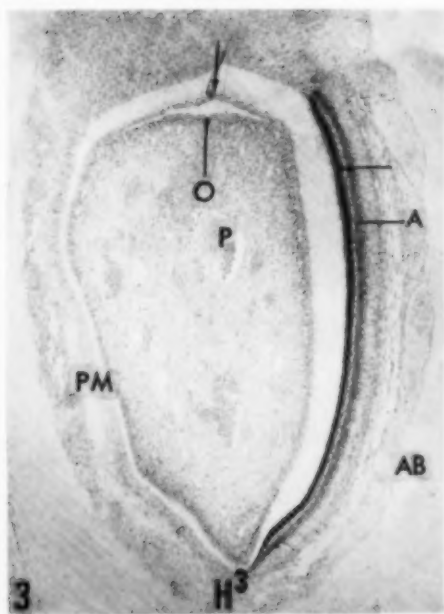
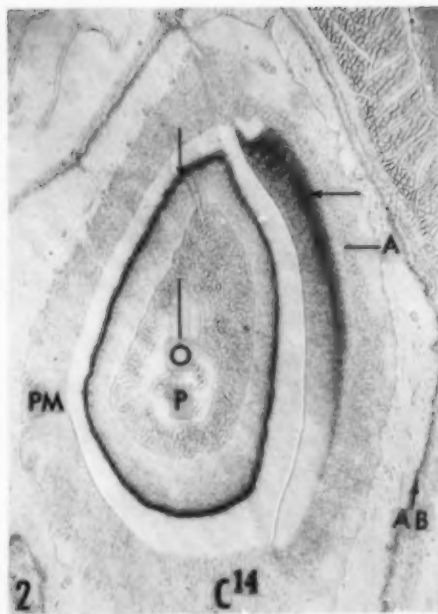
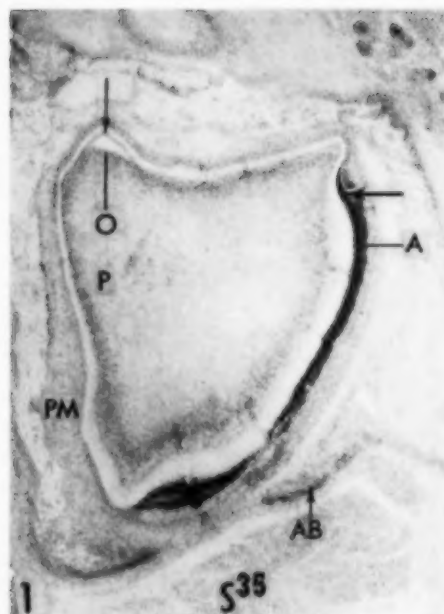
In alveolar bone, a reaction band (AB) is well within mature bone.

FIG. 3. Adult mouse sacrificed 1 hr after methionine- H^3 injection. (Seven month exposure). The results are the same as in Fig. 1.

FIG. 4. Adult mouse sacrificed 7 days after methionine- H^3 injection. (Four month exposure).

Although the tooth is cut obliquely, the dentinal reaction band is clearly visible.

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FIGS. 5 AND 6. Radioautographs of maxillary molars taken from rats injected at 3 days of age with methionine- S^{35} (anterior region at left of pictures). $\times 50$.

FIG. 5. Second molar (unerupted), 6 days after injection. (Haematoxylin-eosin stain. 5 month exposure).

In the enamel organ, the strong reaction (horizontal arrow) is partially obscured by the heavy staining. In the dentine organ, a distinct reaction band is seen over dentine (vertical arrow), while odontoblasts (O) and pulp (P) are unreactive.

FIG. 6. First molar (erupted), 2 months after injection. (5 month exposure).

The enamel has been destroyed by decalcification. In the dentine organ, the reaction band persists, except at the occlusal surface which has been worn away. The intensity of the band and its distance from the dentino-enamel junction remain unchanged.

ELECTRON MICROSCOPY OF UNDECALCIFIED SECTIONS OF HUMAN ADULT DENTINE

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Abstract—Ultra-thin sections of non-decalcified human dentine from recently erupted molars, fixed in 1% osmic acid and embedded in *n*-butyl methacrylate, were cut with a Servall-Porter microtome, equipped with a diamond knife, and examined with the electron microscope.

In predentine and dentine near the pulp, the odontoblast process appears to be a cytoplasmic extension which fills the lumen of the dentinal tubule. In the middle and outer parts of calcified dentine, the odontoblast process takes on a tubular form. In calcified dentine, a peritubular zone surrounds both the tubular and the cytoplasmic parts of the odontoblast process.

The greater degree of electron absorption exhibited by the peritubular zone, together with the observation that selective area electron diffraction shows the presence of apatite, lead to the conclusion that this zone is more calcified than the intertubular substance.

The peritubular zone consists of a fibrillar matrix and inorganic elements, both of which are easily destroyed during routine histological decalcification.

Occasional dentinal tubules show no peritubular zone.

In this investigation an attempt has been made to obtain non-decalcified, ultra-thin sections of normal, adult, human dentine with a view to examining by this direct method the contents of the dentinal tubules and their related structures. Apart from the work of FEARNHED (1957), the only published electron-microscopic studies have been based on indirect replica techniques (SHROFF, WILLIAMSON and BERTAUD, 1954; SCOTT, 1955; TAKUMA, 1958) or on decalcified ultra-thin sections (SCOTT, 1955; SHROFF *et al.*, 1956; YAMAGUCHI, 1957; TAKUMA, 1958).

In previous work (FRANK, 1956, 1957) the author, dealing with the electron microscopic appearance of ultra-thin decalcified sections of human dentine, suggested two variations in the structure of the odontoblast process. Near the pulp this process appeared to fill the lumen of the dentinal tubule completely with what seemed to be a cytoplasmic extension. Further from the predentine the odontoblast process appeared to be tubular and connected by a fibrillar network to the intertubular matrix. The zone occupied by the fibrillar network has been described under various terms on the basis of studies by light microscopy, microradiography (BERGMAN and ENGFELDT, 1954, 1955; MILLER, 1954; BAUD and HELD, 1956) and electron microscopy, such as "translucent area" (BRADFORD, 1950, 1955, 1958), "peritubular translucent zone" (KERÉBEL, 1954, 1958; BLAKE, 1958), "peritubular dentine" (FEARNHEAD, 1957), "calcified canalicular sheath" (SHROFF *et al.*, 1954), "peritubular matrix" (YAMAMOTO, 1957; YAMAGUCHI, 1957; TAKUMA, 1958).

It seemed possible that this very minute zone could be advantageously studied at high magnifications by a direct examination of thin sections containing both organic and inorganic matter *in situ*.

METHOD

Small blocks of normal human dentine were obtained from recently erupted, third molar teeth of young adults. As none of these teeth had reached functional occlusion, they had been unaffected by the forces of mastication. The dentine blocks were fixed in 1% osmic acid for 4 hr (PALADE, 1952), embedded in *n*-butyl methacrylate without previous decalcification and sectioned with a Servall-Porter microtome equipped with a diamond knife (FERNÁNDEZ-MORÁN, 1953). Sections of 200–400 Å in thickness were examined under the electron microscope, model Elmiskop Siemens I. Selective area electron-diffraction studies were also carried out on limited regions of dentine.

RESULTS

For the sake of comparison with the results to follow, Fig. 1 (Plate 1) provides an illustration of a decalcified section of dentine. Here it is noted that the cross-cut, tube-like, odontoblast process (T) is connected to the intertubular substance by a delicate fibrillar network (F).

Non-decalcified, transverse sections of normal dentine show on the other hand the existence of a definite ring-like zone, opaque to electrons, encircling the tube-like odontoblast process and in contact with it. This peritubular zone can be seen in dentine both in the middle and outer portions of this tissue where the odontoblast process assumes a tubular form (Fig. 2, Plate 1) and in the region near the predentine where it takes the form of a cytoplasmic rod (Fig. 3, Plate 1). Sometimes the odontoblast extension is not clearly apparent (Fig. 4, Plate 1).

Lateral branches radiate from the dentinal tubules across the peritubular zone (Fig. 5, Plate 2). These lateral branches possess their own peritubular zone similar to that of the main tubule but smaller in diameter (Fig. 5). The intertubular substance is in continuity with the peritubular zones (Figs. 2, 3, 4). Numerous collagen fibrils, showing typical periodicity, run in all directions across the intertubular substance (Figs. 2, 3, 4). In some areas of well calcified dentine, some of the tubules have no peritubular zones, so that their walls are directly formed by the intertubular substance (Fig. 6, Plate 2).

When comparing undecalcified sections with those obtained after decalcification (Fig. 1), it will be seen that the fibrillar network (F) is normally masked to a considerable extent by mineral salts. That this zone is more highly calcified than the intertubular substance is demonstrated by the fact that it is more electron opaque. Selective area electron-diffraction patterns from this region show ring patterns typical of the apatite group.

DISCUSSION

The comparison of non-decalcified and decalcified, ultra-thin sections by means of the electron microscope suggests that the mineral salts of the peritubular zone are completely soluble in decalcifying solutions. The fibrillar organic matrix of this

zone is also frequently lost during routine histological treatment; thus both the inorganic and organic elements which constitute this zone are easily destroyed by laboratory procedures.

Because the teeth studied were newly erupted and had not been subjected to occlusal stresses, the appearances seen may be presumed to be those of dentine unaffected by age changes or by an environmental stimulus such as attrition. It would appear that the peritubular zone should be regarded as a normal component of young calcified dentine.

Little is known about the nature of the organic matrix of the peritubular zone. It may be similar to that of the intertubular substance and merely impregnated with a larger amount of apatite crystals, or it may have a different biochemical structure altogether. Some histochemical studies (WISLOCKI, SINGER and WALDO, 1948; WISLOCKI and SOGNAES, 1950; SOGNAES, 1955) suggest that the peritubular staining properties differ from those of the intertubular substance. In particular, the peritubular zone is metachromatic, basophilic and stains intensely at low pH, indicating the presence of sulphated acid mucopolysaccharides. At present it is difficult to decide its true nature but, when sections which are sufficiently thin are viewed under the electron microscope, the fibrils of the intertubular substance seem to be in continuity with those of the peritubular zone (Fig. 4; Fig. 7, Plate 2). If the fibrillar material of these two regions is similar in structure, then it is difficult to explain why the matrix of the peritubular zone is so easily destroyed during decalcification. Further work is necessary to clarify this problem. However, there is a definite connexion between the odontoblast process and the fibrillar matrix of both the peritubular zone and the intertubular substance (Fig. 1).

The opportunity of studying the fine structure of non-decalcified ultra-thin sections of hard tissues offers new possibilities of investigating the sub-microscopic anatomy of dentine, and allows more accurate observations to be made on the most resistant type of tissue.

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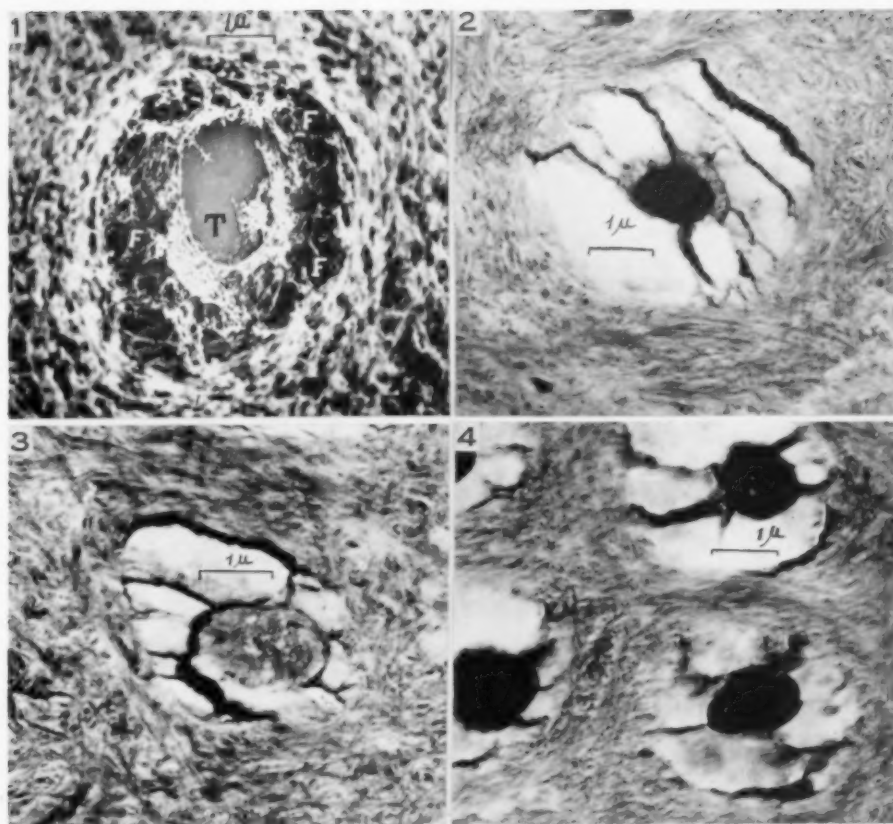


FIG. 1. Decalcified, transverse section through a dentinal tubule. There is a connexion between the tube-like odontoblast process (T) and the fibrillar matrix of both the peritubular zone (F) and the intertubular substance. Compare with Fig. 3. $\times 9500$.

FIG. 2. Non-decalcified, transverse section through a dentinal tubule. A tube-like odontoblast process is in contact with the peritubular zone. Collagen fibrils are present in the intertubular substance. $\times 9000$.

FIG. 3. Non-decalcified, transverse section of a dentinal tubule. The odontoblast process takes the form of a cytoplasmic cord surrounded by a peritubular zone. $\times 10,000$.

FIG. 4. Non-decalcified section through adult human dentine. The lumen of the dentinal tubule is surrounded by the electron-opaque, peritubular zone. The tubule in the bottom right hand corner shows a fibrillar matrix in the peritubular zone, which is orientated in a similar manner to the fibrils of the neighbouring intertubular substance. The fibrils in places show the typical periodicity of collagen. $\times 9000$.

Note: all the electron micrographs are reproduced as facsimiles of the original negatives.

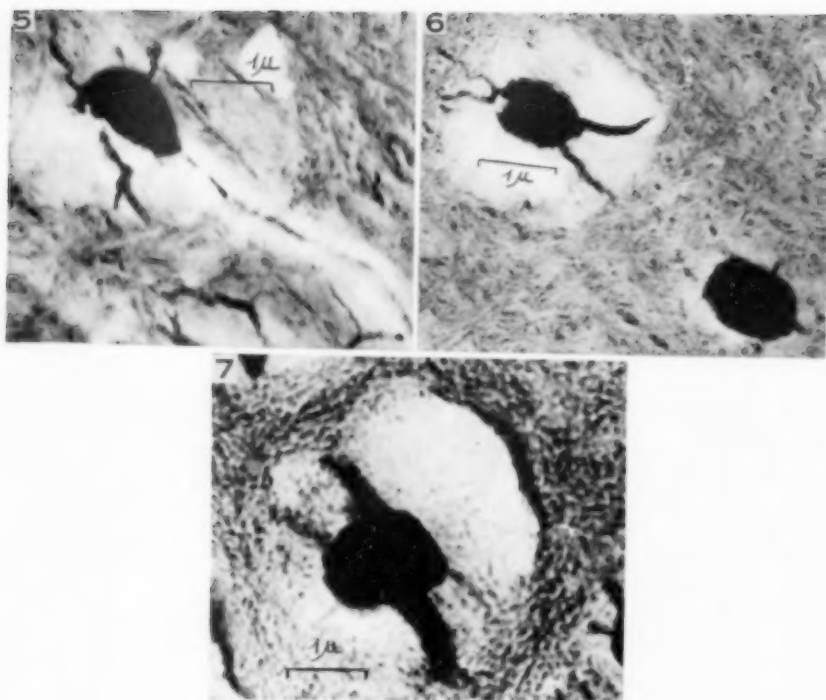


FIG. 5. Non-decalcified, transverse section of human dentine. A lateral branch can be seen to radiate from the internal wall of the peritubular zone, crossing this structure and passing into the intertubular substance where it is surrounded by its own peritubular zone. $\times 11,000$.

FIG. 6. Non-decalcified, transverse section of human dentine. Some of the tubules do not possess a peritubular zone. $\times 11,000$.

FIG. 7. Non-decalcified, transverse section of human dentine. In the peritubular zone a fibrillar matrix can be seen which appears to be connected with that of the intertubular substance. $\times 11,000$.

Note: all the electron micrographs are reproduced as facsimiles of the original negatives.

THE EFFECT OF pH ON THE FLUORIDE INHIBITION OF SALIVARY ACID PRODUCTION

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Abstract—It is confirmed that concentrations of fluoride as low as 1-2 p.p.m. have a detectable inhibitory effect on salivary acid production and that 5 and 10 p.p.m. have a marked effect. The negative results with these concentrations found by LILIENTHAL (1956b) may have arisen from his use of a bicarbonate buffer at pH 6·8 as his incubating medium.

At a pH of 5·0, salivary organisms are more sensitive to fluoride than at neutrality and at this pH concentrations exceeding 6 p.p.m. inhibit acid production completely, so that the pH rises during incubation with glucose. The addition of 230 p.p.m. of calcium ions at pH 5·0 did not prevent the fluoride inhibition.

INTRODUCTION

THE status of the antibacterial theory of fluoride action in caries is still uncertain. Although ISAAC *et al.* (1958) have recently published good evidence for the solubility theory, their results do not exclude the possibility that antibacterial effects may contribute to fluoride action. The results of BIBBY and VAN KESTEREN (1940) on pure cultures of a variety of oral organisms were supported by those of WRIGHT and JENKINS (1954) on whole saliva, in showing that concentrations of fluoride of 1-2 p.p.m. were adequate to produce a detectable, if small, inhibition of acid production. LILIENTHAL (1956b), on the other hand, measuring manometrically acid production by salivary sediment in bicarbonate buffer at pH 6·8, found that 19 p.p.m. was without inhibitory effect and 32 p.p.m. had only a slight action, except in one experiment in which phosphate was added to the medium when 0·5 and 1 p.p.m. were found to inhibit slightly. When calcium and phosphate were present at approximately salivary concentrations, fluoride levels up to 38 p.p.m. were ineffective, although higher levels did inhibit. LILIENTHAL questions, on various grounds, the validity of previous results which suggested that fluoride concentrations as low as 1 or 2 p.p.m. could inhibit.

BOREI (1945) reviewed the factors which had been found to influence the inhibitory effect of fluoride on the respiration of yeast. He reported that the following factors tended to increase the inhibitory powers of fluoride: (1) starving the organisms, (2) allowing fluoride to stand with starved organisms before adding carbohydrate, (3) reducing the pH of the medium, (4) addition of potassium, magnesium or phosphate ions and (5) anaerobic conditions. Of the workers who have studied the fluoride inhibition of saliva, only LILIENTHAL (1956b), in a paper published after the present work had begun, has tested the importance of any of these points and he found that the addition of other ions (calcium, magnesium, phosphate and manganese)

had a significant influence on fluoride inhibition. His use of bicarbonate buffer at a pH of 6.8 as an incubation medium precluded his studying the effect of pH on the inhibitory powers of fluoride.

In view of the different conditions under which LILIENTHAL and the present author were measuring acid production, it was decided to continue the experiments and, in particular, to study the effect of pH on fluoride inhibition.

A note on some of the points developed in the present paper has already been published (JENKINS, 1958).

EXPERIMENTAL

Methods

About 10 ml of saliva were collected from each of twenty subjects approximately 2 hr after their last meal. The samples were pooled, thoroughly mixed by hand shaking and divided into portions which were treated in various ways, depending on the object of the experiment. The final stages of the standard experiment usually consisted of taking 15 ml portions, adding to them varying amounts of a stock solution of sodium fluoride (containing 80 p.p.m. fluoride), along with water if necessary, up to 2.5 ml to give final concentrations over the range 0-10 p.p.m., and 2.5 ml of 20% glucose solution. The mixture was then pipetted into five small test-tubes, each containing 4 ml (equivalent to 3 ml saliva, 0.5 ml fluoride solution and 0.5 ml glucose solution) or in some experiments 2 ml. In the early experiments the same group of tubes was used for determining the pH initially and periodically throughout the incubation. This procedure was found to be unsatisfactory, however, owing to contamination of the saliva with chloride from the potassium chloride bridge of the electrode system, which may stimulate acid production (JENKINS and KENYON, 1959). In later experiments, one tube from each group was taken for checking the initial pH and a pair from each group at intervals during the incubation, the contents being discarded after pH measurement. The pH was determined by means of a Pye pH meter in the later experiments in conjunction with a micro-form of the KCl bridge described by KLEINBERG (1958)—from which chloride contamination was negligible.

The effects of starving salivary organisms and of allowing them to stand with fluoride

The first series of experiments was designed to find whether fluoride was more effective after standing with saliva for some hours and whether the presence or absence of glucose during the standing influenced their fluoride sensitivity. As the results showed that neither factor had an important influence on fluoride action (in agreement with LILIENTHAL's findings) they will not be presented in detail. More work would be required to eliminate the possibility that there were small differences between these groups.

The effect of initial pH on fluoride inhibition

To test whether salivary organisms were more sensitive to fluoride when it was added under acid conditions, part of a batch of saliva was adjusted to about pH 5.0 and was then subdivided into groups containing different concentrations of fluoride

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over the range 1–10 p.p.m. In the first experiments the saliva was acidified with hydrochloric acid but, after the discovery that the chloride ion stimulates acid production, lactic acid was used.

The results of over twenty experiments showed, without exception, that 10 p.p.m. of fluoride when added at a pH of about 5.0 completely inhibited acid production by salivary organisms for several hours, during which the pH rose (Table 1 and Fig. 1a).

TABLE 1. EFFECT OF 10 p.p.m. FLUORIDE ON SALIVARY ACID PRODUCTION AT pH 5.15

	pH			
	0 hr	2 hr	3½ hr	24 hr
Control	5.15	4.90	4.65	3.97
Fluoride	5.14	5.35	5.42	4.32

Note: Each figure is the mean pH of four tubes incubated together.

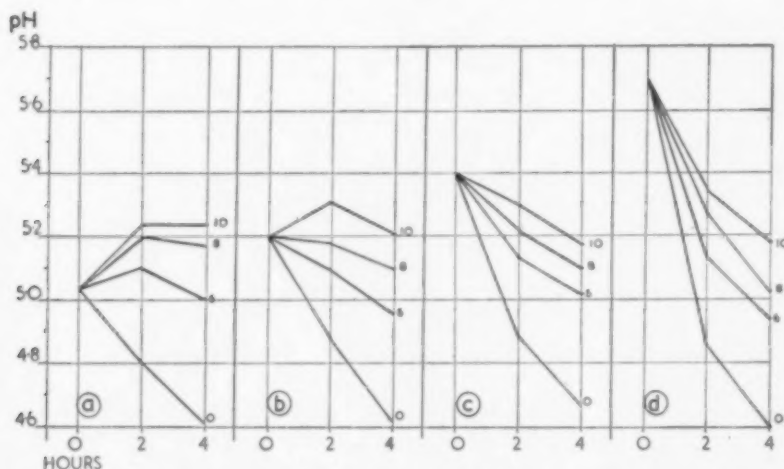


FIG. 1. Effect of adding 0, 6, 8 and 10 p.p.m. of fluoride to saliva-glucose mixtures at initial pH values of (a) 5.04, (b) 5.2, (c) 5.4 and (d) 5.7.

The next series of experiments was designed to determine (1) the minimum concentration of fluoride necessary for this complete inhibition of acid production and also (2) over what range of initial pH complete inhibition could be demonstrated.

Pooled saliva was divided into four batches and adjusted to pH values of 5.0, 5.2, 5.4 and 5.6–7 with lactic acid. Each batch was then subdivided and fluoride added to give final concentrations of 0, 6, 8 and 10 p.p.m. The procedure from this point was similar to that of the previous experiments.

The results (Fig. 1) showed that when the initial pH was 5.0, all three concentrations prevented a further fall in pH for at least 2 hr. At 5.2, only the two higher levels

inhibited completely, and at 5.4 and 5.7 none of the fluoride concentrations completely prevented a fall in pH, although powerful inhibitions were, of course, still present. It must be concluded that over the range 5-5.7, pH is an important factor in controlling the effectiveness of fluoride inhibition. Five experiments of this type were carried out and they all gave extremely consistent results.

The next point investigated was the possibility that, even when fluoride was added to neutral saliva, it did not exert its inhibitory effect until the pH had fallen to the region of 5.0. If this occurred, then there would be no difference between the effect of adding the fluoride at either pH 5.0 or pH 7.0.

Incubation mixtures were set up, with and without 10 p.p.m. fluoride, at about neutrality and the pH determined at frequent intervals throughout the incubation, thus making it possible to find at what pH the inhibition began and to compare its intensity at various pH values. The results (Fig. 2) showed that fluoride undoubtedly

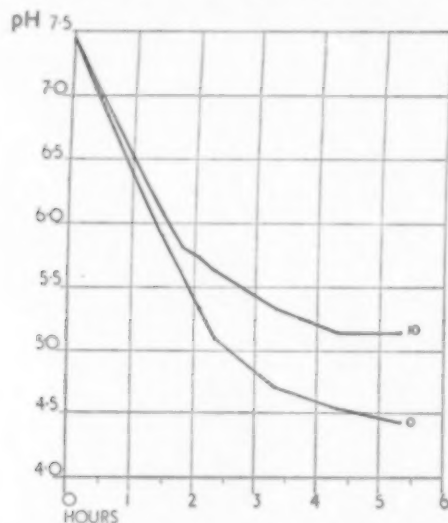


FIG. 2. Serial readings on saliva and glucose incubated with and without 10 p.p.m. fluoride from initial pH of 7.5.

inhibits even above a pH of 6.0, but that the inhibition becomes more marked at about pH 5.0 when acid production in the presence of fluoride almost ceases. There was, however, never any tendency in these experiments for fluoride to make the pH rise when it reaches about 5.0 and this suggests that the organisms are more sensitive to fluoride when, as in the experiments exemplified by Table 1 and Fig. 1(a) and (b), they are exposed to fluoride only after the pH has fallen.

However, another possible explanation presents itself. This arises from the fact that the experiments described above, showing the greater sensitivity of oral bacteria to fluoride at pH 5.0, were conducted on saliva whose pH had been adjusted by the addition of acid. The bacteria in such saliva are, of course, in a different condition from those in saliva which has become acid as a result of metabolizing carbohydrate.

The effect of adding fluoride to saliva during active metabolism

The effect of fluoride added to saliva which had reached a pH of 5.0 as a result of incubation was tested as follows. Saliva was taken, divided into batches of 15 ml, and 2.5 ml of glucose solution added to each and the mixtures incubated. One tube had 2.5 ml of 80 p.p.m. fluoride added, and another 2.5 ml of water, at the beginning of the incubation. After incubation for 2½ hr, by which time the pH had reached about 5.5, 2 ml samples were withdrawn at intervals from the fluoride-containing tube and its control and the pH determined. When the readings of this "pilot" control tube showed that the pH was 5.2, 2.5 ml of fluoride solution, warmed to 37°C, were added to another 15 ml batch of saliva and the same volume of warm distilled water was added to yet another. Samples were withdrawn from both tubes immediately and every hour, to follow pH changes. The procedure was repeated on another pair of tubes when the pH of the pilot tube reached 5.0. Thus, with a minimum of disturbance, fluoride was added to the metabolizing salivary bacteria making possible a comparison, under more physiological conditions, between the effects of fluoride when added at pH 7 or 5.

The results showed that under these conditions, the addition of 10 p.p.m. of fluoride completely arrested the fall in pH for some hours, if it was added when the pH was close to 5.0. A rise in pH occurred sometimes with 10 p.p.m. but it was much smaller than in previous experiments (Fig. 3), although unequivocal with 20 p.p.m. Comparison of the slopes of the curves in Fig. 3 shows that even if complete inhibition did not occur, as when the fluoride was added at 5.4, a pH too high for

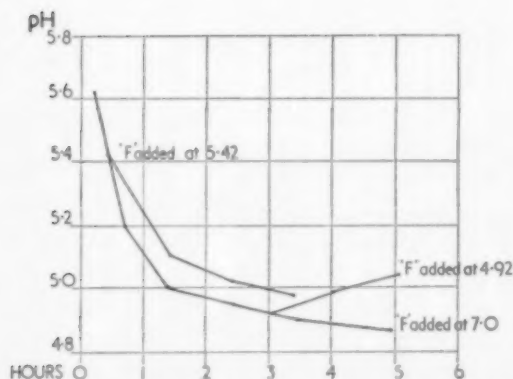


FIG. 3. The effect of adding 10 p.p.m. of fluoride to saliva at different pH values during incubation with glucose. These curves were produced by measuring pH in three separate tubes of saliva incubated to the pH values shown before fluoride was added. The control curve (without fluoride) is not included.

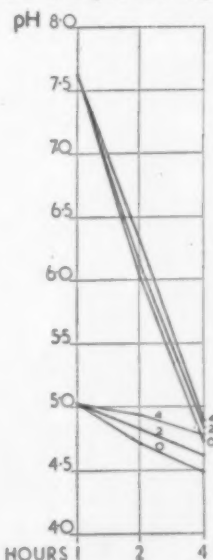


FIG. 4. The effect of 0, 2 and 4 p.p.m. of fluoride added to pH values of 7.6 and 5.0 to saliva-glucose mixtures.

it to stop acid production completely under these conditions, the inhibition was greater than at similar pH values in mixtures incubated with fluoride from neutrality.

The effect of 2 and 4 p.p.m. of fluoride

Since LILIENTHAL questioned the validity of results showing the inhibitory effects of very low concentrations of fluoride, experiments were carried out in which the effect of 0, 2 and 4 p.p.m. of fluoride were tested in saliva with initial pH at about neutrality and at 5.0. Samples were taken at intervals chosen to test the activity of these concentrations at pH values between 7.0 and 5.0. The results (Fig. 4) confirmed that both levels were active even above pH 6.0 and also that the mixtures incubated from 5.0 were more sensitive than those at 7.0. The differences after 4 hr incubation between the final pH values of the controls and mixtures with 4 p.p.m. were: starting at 7.0—0.19, 0.23, 0.17, 0.22, 0.09, 0.16 (average 0.18); starting at 5.0—0.31, 0.31, 0.20, 0.32, 0.21, 0.26 (average 0.27).

The effect of pH on fluoride inhibition of salivary sediment

Since salivary sediment presumably resembles plaque more closely than saliva and since HARGREAVES and MANLY (1956) have reported that higher concentrations of inhibitors are needed to influence sediment than saliva, it was decided to compare the effects of fluoride at pH 5.0 on these two materials.

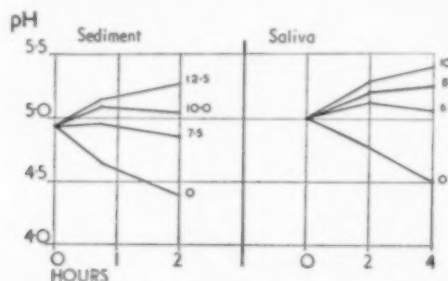


FIG. 5. Comparison of various levels of fluoride on pH changes during incubation with sugar of saliva or salivary sediment.

The results (Fig. 5) showed that sediment was only slightly less sensitive than saliva. The small difference observed probably arose largely from the dilution of the added fluoride by the water present in the sediment.

Effect of other ions on the fluoride inhibition

Preliminary experiments with magnesium and phosphate additions to saliva failed to show any consistent effect on fluoride inhibition. In a second group of experiments, the influence of the addition at pH 5.0 of final concentrations of 230 p.p.m. calcium, 31 p.p.m. magnesium and phosphate (=400 p.p.m. P) was tested on inhibition by 6, 8 and 10 p.p.m. of fluoride. The results (Table 2) show that in all

TABLE 2. EFFECT OF CALCIUM, MAGNESIUM AND PHOSPHATE IONS ON FLUORIDE INHIBITION OF ACID PRODUCTION BY SALIVA ADJUSTED TO pH 5.0

Concentration of F	pH after incubation (average of 7 experiments)							
	Without Ca, Mg, P				With Ca, Mg, P			
	0	6	8	10	0	6	8	10
3 hr incubation	4.65	4.93	4.98	5.01	4.59	4.86	4.90	4.92
5-6 hr incubation	4.29	4.87	4.97	5.03	4.31	4.77	4.83	4.88

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mixtures containing the additional ions, including the control with no fluoride, the pH was slightly lower than in the corresponding mixtures without the addition. At 3 hr, the effect on the fluoride inhibition is negligible but becomes larger after 5-6 hr incubation. It is quite clear that high concentrations of calcium do not, at pH 5.0, prevent the action of fluoride in saliva—a result which differs from that obtained by LILIENTHAL with his technique.

DISCUSSION

The results in this paper confirm those of WRIGHT and JENKINS (1954) that salivary acid production can be inhibited by concentrations of fluoride as low as 2 and 4 p.p.m. Few of the present experiments have tested 1 p.p.m. and none 0.5 p.p.m., but by extrapolation the previous conclusions are given excellent support. There are at least four differences which could conceivably have explained the discrepancy between the present findings and the substantially negative results of LILIENTHAL with concentrations of fluoride lower than 19 p.p.m. First, LILIENTHAL's experiments were on salivary sediment whereas most of the present work was on saliva. However, the discrepancy cannot be explained on this basis, for the direct comparison of the sensitivity to fluoride of these two media showed very little difference. Secondly, in LILIENTHAL's experiments the cells were incubated in bicarbonate buffer at pH 6.8 under anaerobic conditions. This variation in pH probably played a part in the difference but is unlikely to explain the whole of it, because the inhibitory effect of as little as 2 and 4 p.p.m. of fluoride, although smaller than at lower pH, was consistently detected between pH 6 and 7, even in aerobic conditions. The anaerobic environment used by LILIENTHAL would be expected, according to BOREI, to increase sensitivity to fluoride. The third difference was that magnesium and phosphate ions (salivary constituents found by WARBURG and CHRISTIAN (1942) to enhance fluoride inhibition) would be greatly diluted by the bicarbonate buffer and virtually absent in some of his experiments in which he states that the cells were washed with distilled water. The absence of these ions seems a plausible explanation of at least part of the difference between the results. It was after the addition of phosphate that LILIENTHAL observed his only positive results with 1.0 and 0.5 p.p.m. fluoride. The

fourth possibility is that manometric methods are less sensitive than pH determinations as a measure of small differences in acid production. This explanation is made less likely by the finding just mentioned, that on at least one occasion (LILIENTHAL does not state how many) the inhibition by 1.0 and 0.5 p.p.m. of fluoride was detectable.

The next point to consider is the nature of the marked and consistent rise in pH observed when saliva was incubated with fluoride at an initial pH of 5.0. This rise was never observed in saliva incubated with fluoride from neutrality, and it was thought at first to be an artifact caused by the unnatural conditions arising when saliva was incubated from an initially low pH. Complete inhibition and sometimes a small rise in pH did occur, however, in the experiments in which 10 p.p.m. of fluoride were added while the organisms were actively metabolizing, and with higher concentrations of fluoride a rise occurred consistently. It is concluded, therefore, that the rise in pH is an event that could occur under physiological conditions if a sufficiently high fluoride concentration were present at a pH near 5.0. This experiment also provides good evidence that the pH at which salivary organisms are first exposed to fluoride influences their sensitivity, since the rise in pH occurred only when the fluoride was added under acid conditions. This conclusion is confirmed by comparing the average inhibitions of 2 and 4 p.p.m. when added at pH 5.0 and at 7.0.

It is suggested that if acid production is, for any reason, proceeding slowly (as it does at the unfavourable pH of 5.0) then lower concentrations of fluoride are sufficient to stop it completely compared with those required when acid production is favoured. When acid production stops, the alkali-producing mechanisms of saliva are unopposed and consequently the pH rises.

The relevance of these observations to the caries process

In order to assess the antibacterial theory of fluoride action it is necessary to know (1) the minimum concentration of fluoride which can inhibit acid production and (2) the fluoride concentrations present in the plaque under various conditions. As this work was proceeding HARDWICK, FREMLIN and MATHIESON (1958) have been developing and applying methods of measuring the fluoride content of plaque, including that which may enter it from the outer enamel surface. While their work is still in progress it is impossible to come to any final conclusion about the relevance of the present results. It can only be pointed out that if fluoride does enter plaque from the enamel surface, and if the concept of a critical pH be accepted, then it would not reach the organisms until the pH fell to a value which, the present work suggests, favours its inhibitory action. It seems possible that further acid production beyond the critical pH might be greatly reduced or even prevented altogether in the inner plaque by a sudden influx of as little as 6 p.p.m. of fluoride. Even lower concentrations would have some inhibitory effect. It is probable that quite small absolute changes in the pH curve of plaque between 5.0 and 5.5, may influence greatly the amount of decalcification. For example, if the critical pH of a particular plaque was 5.3, then a difference between a final pH of 5.2 and 5.3 is the difference between some decalcification and none at all.

The experiments of LILIENTHAL (1956a) on the effects of powdered enamel topically applied with fluoride, synthetic hydroxyapatite and natural fluorapatite on acid production by salivary sediment at pH 6.8, led him to conclude that the fluoride of the enamel surface could not influence acid production. He based his calculations on the aqueous solubility of calcium fluoride at neutrality, however, whereas the relevant point is its solubility in plaque at pH 5.0, which is not known. The fact that caries occurs at all shows presumably that the outer layer of the enamel is capable of dissolving in plaque and the experiments described in this paper show that fluoride can remain active in saliva in the presence of more than a twenty-fold excess of calcium at pH 5.0. Although it is admitted that the whole question is speculative, LILIENTHAL's grounds for rejecting this concept do not seem to be conclusive.

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EXPERIMENTAL DENTAL CARIES IN GOLDEN HAMSTERS—IX

THE EFFECT OF A CARIOGENIC DIET FED AT INTERVALS

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Abstract—The influence on the caries incidence of different ways of feeding a cariogenic diet to hamsters was studied. In four preliminary experiments the following observations were made.

The animals did not eat sufficient for normal growth if the food was supplied only at three half-hour mealtimes each day.

A cariogenic diet containing 62 per cent sucrose did not cause any carious lesions if it was fed at three meals a day and during the weekend period or for 3 days a week either every second day or on three consecutive days. If it was given every day it had a marked cariogenic effect.

When the amount of sucrose consumed during these 3 days a week was distributed over the whole week, by feeding a diet containing 26 per cent sucrose, a significant amount of caries developed.

THERE are several factors other than the constituents of a cariogenic diet that influence the degree of dental caries produced by such a diet. It has been shown that the age at which the diet is introduced, the fluidity of the diet, the amount of diet supplied and many factors in the actual maintenance of the animals are of importance (GUSTAFSON *et al.*, 1955). It would therefore be of interest to ascertain if a change in the manner in which the experimental animals consume their food has any influence on its cariogenic effect. This would be of special value if the habits were changed so as to resemble those of civilized human beings by limiting feeding to "meal times".

When food is given *ad libitum*, rats and hamsters normally eat small amounts almost continuously day and night. In experiments on caries this is the usual way of feeding the various diets. The frequent ingestion of cariogenic food renews the food deposits round the teeth and these are constantly available for bacterial breakdown with acid production. This might be expected to increase the cariogenic effect of the diet.

Since 1952 this problem has been investigated from various points of view in four series of pilot experiments with golden hamsters from our stock of animals.

EXPERIMENTAL METHOD

In each experiment synthetic diets were used and introduced immediately after weaning at 21 days of age. As the experiments were of a preliminary nature only one experimental period of 150 days was used. At the end of this period the animals

were decapitated and their teeth examined. The recording and scoring was made according to the method previously described (GUSTAFSON, STELLING and BRUNIUS, 1952). In this method, only the lower third and upper second and third molars are examined and only lesions with a size of five units or more recorded. The lesions were outlined on special scorecards and their area measured with a planimeter, calibrated to express the circumscribed area directly as a percentage of the area of the tooth that can be attacked. Instead of expressing the carious areas in percentages the term "cariou units" is preferred.

The composition of the cariogenic diet was as follows:

Casein	20 g
Sucrose	65 g
Arachis oil "ADE"	7 g
Cellulose flour	3 g
Salt mixture "6"	4 g
Vitamin mixture "20" with cystine	0.8 g
Vitamin mixture "22"	0.2 g

Salt mixture "6":

NaCl	43.3 g
MgSO ₄ ·H ₂ O	76.5 g
NaH ₂ PO ₄ ·H ₂ O	86.8 g
K ₂ HPO ₄	238.5 g
CuSO ₄ ·5H ₂ O	1.25 g
Ca(H ₂ PO ₄) ₂ ·H ₂ O	135.0 g
Calcium lactate	325.0 g
Ferric citrate	29.5 g
KI	0.125 g
MnSO ₄ ·H ₂ O	3.79 g

Arachis oil "ADE":

DL α -Tocopherol	1 g
Vitamin A	1,600,000 I.U. (of synthetic all-trans vitamin A-acetate)
Ergocalciferol	160,000 I.U.
Arachis oil	to 1400 g

Vitamin mixture "20" with cystine:

Choline chloride	120 g
L-Cystine	30 g
Inositol	60 g
p-Aminobenzoic acid	30 g
	<hr/>
	240 g

Vitamin mixture "22":

Thiamine mononitrate	1.50 g
Riboflavin	1.50 g
Nicotinamide	2.25 g
Pyridoxine hydrochloride	1.50 g
Calcium-D-pantothenate	1.50 g
Pteroylglutaminic acid	1.20 g
Biotin	0.01 g
Cyanocobalamin-sodium chloride, 1 in 1000	3.00 g
"Menadiol" sodium phosphate	0.90 g
Ascorbic acid	6.00 g
Wheat starch	40.64 g
	<hr/> 60.00 g

The only difference in the non-cariogenic diet was the substitution of an equal weight of wheat starch for the sucrose.

Experiment 1 (1952-1953)

From our stock of hamsters, forty-nine newly weaned animals were distributed, as far as possible with regard to litter and sex, in two equivalent groups, one experimental (17 ♂ and 8 ♀) and one control (17 ♂ and 7 ♀). Both groups were fed the cariogenic diet described above. The animals of the experimental group were given their food-cups three times a day at 8.30 a.m., 12.30 p.m. and 3.30 p.m. The cups were removed after half an hour each time. The animals were given no food at night. For practical reasons a compromise arrangement was made over weekends. The last meal on Saturdays was given at 1 p.m. and this food was left in the cages till 9 a.m. the following Monday morning. In the control group, the animals had access to their food day and night and thus could eat whenever they liked.

Experiment 2 (1953-1954)

In this experiment seventy-one hamsters were used, divided into two groups. The experimental group (19 ♂ and 16 ♀) was given the same diet at three meals a day and during the weekend period as in experiment 1 but, for reasons discussed later, during the night they were given food-cups with the non-cariogenic diet described above. The control group (19 ♂ and 17 ♀) was fed the same caries-producing diet in the same way as in experiment 1.

Experiment 3 (1954-1955)

Four groups with 140 animals in all were included in this experiment.

Group a (16 ♂ and 19 ♀). The cariogenic diet was fed exactly as in experiment 2 as three meals a day and during the weekend period and was also given the non-cariogenic diet at night.

Group b (17 ♂ and 18 ♀). The cariogenic diet was given to this group day and night on Mondays, Wednesdays and Fridays and the non-cariogenic diet for the rest of the week.

Group c (15 ♂ and 19 ♀) and *Group d* (17 ♂ and 19 ♀). These groups were controls and were fed the cariogenic and the non-cariogenic diets respectively in the normal way.

Experiment 4 (1957-1958)

Only male hamsters were used in this experiment. They were distributed into five groups of 33-35 animals.

Group 1. This was fed with the cariogenic diet as in experiment 3, group a.

Group 2. The same diet was given on Mondays, Tuesdays and Wednesdays and the non-cariogenic for the rest of the week.

Group 3. This group was given a cariogenic diet each day and night in which the content of sucrose was calculated to correspond to the amount of sucrose consumed during the three weekdays in groups 1 and 2 distributed over a whole week. Assuming that the animals eat approximately the same quantity of food each day on the cariogenic or the non-cariogenic diet, this would correspond to a diet of 26 per cent sucrose. A diet with that percentage of sucrose was obtained by mixing 857 g of the cariogenic diet used with 1143 g of the non-cariogenic diet.

Groups 4 and 5. These were control groups on the cariogenic and non-cariogenic diets respectively.

RESULTS

Growth. The different groups within each experiment showed very nearly the same growth except in experiment 1, in which the animals of the experimental group grew very slowly with a final weight of about only one-half of that of the control animals (cf. Fig. 1). The cause of this poor growth is discussed later.

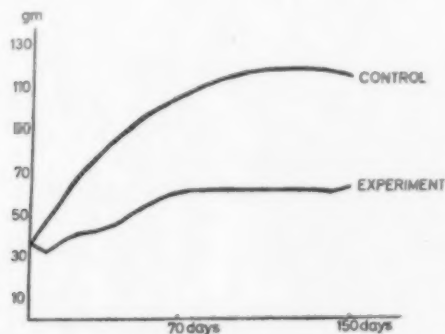


FIG. 1 The weight curves in experiment 1.

Carious lesions. The incidence of caries in the various groups of the experiments is shown in Table 1.

TABLE 1.

Experiment	Group	Cariou units
1	Experimental	0.2±0.2
	Control	53.3±4.1
2	Experimental	0
	Control	45.6±3.8
3	a	0.3±0.2
	b	0
	c	36.9±5.5
	(control)	
	d	0
4	(control)	
	1	0
	2	0
	3	7.8±1.8
	4	36.6±3.5
	(control)	
	5	0
	(control)	

It is evident that the incidence of caries on the cariogenic control diets varies between 53.3 and 36.6 units and on the non-cariogenic diet no carious lesions are recorded in any group. The experimental groups on the various food regimes developed practically no caries at all except group 3 in experiment 4, where a significant amount of caries (7.8 ± 1.8 units) was to be seen.

DISCUSSION

From experiment 1 is evident that the attempt to feed hamsters on three meals a day was followed by a markedly slow growth. This must obviously be due to partial starvation. The experimental animals were not able to change their eating habits and did not use the short time they had access to the food to eat enough of it. During the weekend periods they could eat according to their natural habits and that probably prevented their death by starvation. As appears from Table 1 no caries developed on this food regime. As to the cause of this result no definite conclusion can be drawn from this experiment alone. Many observations support the theory that a restricted food supply is associated with a decreased incidence of caries and this may possibly be the case here. The starvation factor was eliminated in experiment 2 by feeding the non-cariogenic diet at night and both groups now showed the same growth. The result was, however, the same; no caries developed on the experimental regime. Now it may be that the consumption of the sucrose

diet was even less than in experiment 1, as the animals could not have been so hungry on Saturdays when they were given their food-cups with this diet as they must have been in the earlier experiment. Of course it would have been of value to have been able to measure the amount of food consumed of each of the diets but it was not found possible to do so either in this or in the subsequent experiments. A repetition of this feeding regime a year later, in experiment 3, group a, confirmed the result. Thus it has been shown that feeding a cariogenic diet with 65 per cent sucrose during the weekend period, and in small quantities at meals during the week, was evidently not sufficient to cause any carious lesions.

In experiment 3, group b, another method of intermittent feeding was introduced. The animals in this group were given the cariogenic diet night and day every second day and the non-cariogenic diet on the days between and over the weekend period. Although this regime markedly increased the time available for acid formation in the mouth compared with the potential time in the earlier experiments, no caries developed. The result was confirmed by a repetition in experiment 4, group 1. This great difference between the caries-producing effect of a diet fed, alternately with a non-cariogenic diet, 3 days a week and the same diet fed the whole week might be explained by assuming that a remineralization of the initial lesion takes place during the intermediate days when the non-cariogenic diet was fed. If this is the case, there would probably be a difference in cariogenic effect if the diet was fed for 3 consecutive days every week. That regime was fed in group 2, experiment 4, but even then no carious lesions developed. But there is another possibility to explain the absence of caries in these experimental groups. The amount of sucrose consumed during these 3 days may be too small to be caries-conducive. The aim with group 3 in experiment 4 was to study if the amount of sucrose consumed during these days every week was sufficient to cause caries if it was distributed over the whole week. The construction of such a diet is described above and its content of sucrose was 26 per cent. Now a significant degree of caries developed with an average of 7.8 units. The result thus showed that the decisive factor in the non-cariogenic effect of the 3 day consumption of a sucrose diet was not the smallness of the amount of sucrose consumed but the way in which it was consumed. The results, at least under the conditions present in these experiments, also lend support to the theory that the cariogenic effect of a sucrose diet is predominantly exerted in the mouth and not via nutritional channels.

If these experiments are continued and the number of days in the week for which the cariogenic diet is given is increased, they will show how many days a week it is necessary to eat this type of diet to have caries. It is likely to be about 4 or 5 days as 7 days gives a very high incidence of caries.

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HUMAN PAROTID SALIVA AS A SOLE SOURCE OF NUTRIENT FOR MICRO-ORGANISMS*

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Abstract—Parotid saliva, collected under aseptic conditions from human beings, was studied as the sole source of nutrients for strains of *Aerobacter cloacae*, *Bacillus cereus* var. *terminalis*, *Bacillus subtilis* var. *niger*, *Candida albicans*, *Corynebacterium diphtheriae*, a *Lactobacillus* sp., *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus pyogenes*, and a viridans streptococcus. The populations of staphylococci, enterococci, *Aerobacter*, yeasts, and spores and vegetative cells of *B. subtilis* and *B. cereus* tended to survive and multiply suboptimally in the parotid saliva. Unfavourable effects of the reduced hydrogen-ion concentration of collected parotid saliva on the staphylococci, enterococci and *Aerobacter* were less when the pH was adjusted with phosphate buffer. Germination of spores, multiplication of vegetative cells and sporulation of the bacilli occurred in thirteen of fifteen parotid salivas. Eight salivas delayed germination, multiplication or sporulation from 24 to 96 hr of incubation. The populations of the lactobacillus, β -streptococcus, viridans streptococcus and diphtheria bacillus tended to be unable to survive and multiply even though the pH of the saliva was altered by addition of a phosphate buffer. Differences between the effects of the saliva samples on bacteria were also noted with the strain of *Aerobacter* and lactobacillus, otherwise parotid salivas from several human beings had similar effects on microbial growth.

THE secretions of the human oral cavity are derived from three pairs of major salivary glands together with small mucous glands randomly distributed in the mucous membrane. Although the amount of saliva delivered into the oral cavity by the parotid glands is much lower than that from the combined secretions of the sublingual and submaxillary glands (SCHNEYER and LEVIN, 1955), parotid saliva is considered as having an important influence on the oral environment. The parotid saliva contains a high concentration of β -amylase and several other enzymes (CHAUNCEY, LIONETTI, WINER and LISANTI, 1954), whereas the submaxillary-sublingual saliva has lower concentrations of β -amylase, but high amounts of mucopolysaccharides. The relative concentrations of the several constituents of parotid saliva have been noted to change according to the rate of flow (BRAMKAMP, 1936).

A number of workers have studied the use of human parotid or whole saliva as a supplement in artificial culture medium. WEISBERGER (1946) reported that whole saliva would replace the mineral salts required in a synthetic medium for an oral lactobacillus. It was necessary to concentrate the saliva before it could be substituted for thiamin hydrochloride, calcium pantothenate or nicotinic acid in the medium.

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Even then the growth was not optimal. Autoclaved whole saliva supported the production of toxin by diphtheria bacilli (TASMAN and SMITH, 1953). UMEMOTO and associates (1950a and b) collected human parotid saliva under aseptic conditions. Saliva, not found to contain viable microbes, was added to culture media. The proportions of saliva to medium were not the same for optimal growth of the several microbes under study. Although reports of the above kinds appear frequently, little or no work has been reported on the use of either parotid or submaxillary and sublingual saliva as the sole source of microbial nutrients. Thus, our immediate goal was to determine whether human parotid saliva, collected aseptically, could serve as the sole source of nutrient for a number of microbes commonly isolated from the mouth.

MATERIALS AND METHODS

(1) *Saliva collection*

Human parotid saliva was collected by applying a sterile cup (CURBY, 1953) to the treated orifice of Stenson's duct and maintaining it in position with negative pressure. Sterile plastic Tygon tubing, attached to the cup, carried the saliva to a suitable sterile container. The collecting cup and tubing were disinfected immediately after each use. The cup was exposed to benzalkonium chloride, U.S.P. (1:1000 in sterile distilled water) for 24 hr and then rinsed in sterile distilled water for 2-3 hr to remove residual chemical. The plastic tubing was wrapped separately in aluminium foil and autoclaved at 121°C for 10 min. The tubing was attached aseptically to the cup just prior to use. The cheek was kept retracted throughout the entire procedure of "disinfecting" the orifice of Stenson's duct and applying the apparatus. The orifice was first dried with a sterile swab, then an iodine antiseptic was applied. The antiseptic was removed, after about 30 sec, by wiping the orifice with a sterile swab saturated with 70% ethanol (by volume). The orifice was then wiped with a sterile dry swab. The aluminium foil was removed from the receptacle end of the collection tube and the tubing inserted into a sterile test-tube which was then replugged. The flow of parotid saliva was increased by giving the patient lime- or lemon-flavoured troches. Collection of the saliva was continued until the test-tube was almost filled (22-25 ml). The time required to collect the desired amount of parotid saliva varied greatly from patient to patient. Collections were made about 2 hr after breakfast or 2 hr after lunch.

The pH of the saliva was determined electrometrically immediately after collection and again after 24 hr incubation at 37°C. Tests for viable organisms in the incubated saliva were done as follows: 0.1 ml was spread on each of two blood-agar plates (5 per cent sterile horse blood in Difco blood-agar base); one incubated anaerobically, the other aerobically at 37°C for 3-4 days. No growth on either of the blood-agar plates indicated that the saliva could be used in the growth experiments. Such saliva was transferred aseptically to sterile vaccine bottles and stored at 5°C until used.

(2) *Growth curve studies*

(a) *Standardization of inoculum.* The standardization was based on work with *Staphylococcus aureus* No. 44A (obtained from William Pepper Laboratory, University

of Pennsylvania Hospital). After 24 hr incubation in nutrient broth (Difco) at 37°C, the culture was washed with three changes of sterile 0.85% NaCl in distilled water. The final dilution was such that a turbidity reading of 100 was obtained using a Klett-Summerson colorimeter (filter with wavelength 400-465 m μ).

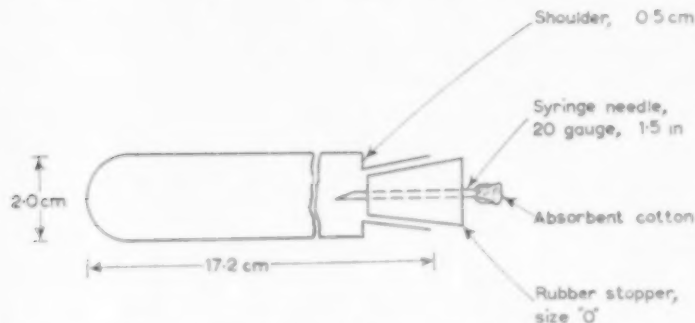


FIG. 1. Schematic diagram of the modified test-tube, referred to as the "roller tube".

Three millilitres of the sterile saliva was put into each of two "roller tubes" (see Fig. 1). The "roller tube" is a Pyrex test-tube 17.2 cm long by 2.0 cm in diameter which has been modified by shaping the open end to form a shoulder 0.5 cm wide and a conical opening which would receive an "O" rubber stopper. A sterile hypodermic needle (20 gauge; 1½ in. long) containing cotton in the lock end was passed through the length of the stopper to provide an airway. The saliva in one of the tubes was inoculated with 0.01 ml and the other tube received 0.1 ml of the diluted culture. Incubation was at 37°C in an apparatus which held the tubes in a horizontal position and rotated at 26 r.p.m. The use of the roller tubes and machine was an attempt to provide the greatest possible surface-volume ratio.

Samples were removed from each tube at 24 and 48 hr and inoculated in appropriate dilution into Difco nutrient agar medium (pour plates). Inocula were 0.1 ml aliquots undiluted, and diluted to 10^{-1} , 10^{-2} and 10^{-3} in isotonic saline. The plates were incubated for 48 hr before the colonies were counted. Colonies were picked at random from the plates to prepare smears for staining by Gram's method and to identify by typing with the homologous phage (BLAIR and CARR, 1953).

(b) *Procedure for growth curve studies.* For each study, a total of 12 ml of a single saliva sample was used. A 3 ml aliquot was dispensed aseptically to each of four sterile tubes stoppered as described above. All tubes were inoculated with 0.1 ml of washed 24 hr culture (unless specified otherwise) of turbidity described above. Incubation was done simultaneously at 37°C and a different tube was removed at each plating period except at 48 and 72 hr. This was done to prevent the reduction in volume created by sampling during each of the early culture periods. The same tube was used for 48 and 72 hr samplings because it became apparent that changes in volume had little effect at this late stage in the growth curve. Controls of isotonic saline and culture broth were prepared and treated in the same way.

Culture periods were 0, 6, 24, 48 and 72 hr. Four dilutions (made with isotonic saline) of all saliva samples were cultured; two pour plates per dilution. All plates were incubated at 37°C aerobically for a period of 3 days. The dilutions and the culture medium used for each bacterium differed.

The pH of the contents of each tube was determined at 0, 6, 24, and 72 hr. The 48 hr measurement was not made so as to prevent further volume change. The effects of hydrogen ion concentration on microbial growth were studied in several ways. First, the optimum pH for growth of each microbe was determined in the appropriate culture medium. Secondly, growth studies were made in parotid saliva without pH adjustment. Lastly, phosphate buffer was added to a final concentration of 0.045 M. Table 1 presents pH measurements.

Antibacterial activity of each saliva was determined against each microbe to be used in the culture tests. Wells were cut in suitable agar medium (the bottom of each well being sealed with melted and cooled agar) which had been seeded with the specific bacterium. About 0.05 ml of each saliva sample was used per well. Suitable control fluids were also used. In no instance did the saliva inhibit the growth of the test organisms.

Separate growth studies were carried out, in the manner described above, using ten species of microbes, many of which were isolated from the oral cavity. The identity of each culture was checked, after exposure to saliva, as representing the inoculated cells. Phage typing was employed when applicable. The microbes and the culture media employed were as follows:

<i>Aerobacter cloacae</i>	Nutrient agar (Difco)
<i>Bacillus subtilis</i> var. <i>niger</i> strain No. 51-8	Nutrient agar (Difco)
<i>Bacillus cereus</i> var. <i>terminalis</i>	Nutrient agar (Difco)
(Supplied by HALVORSON, University of Wisconsin)	
<i>Corynebacterium diphtheriae</i> strain No. 48-1	Brain-heart infusion agar (Difco)
<i>Lactobacillus</i> strain No. 5JC	SL medium (Difco)
<i>Staphylococcus aureus</i> strain No. 44A	Trypticase soy agar (BBL)
<i>Streptococcus haemolyticus</i> strain No. 51-5	Blood-agar base (Difco)
<i>Streptococcus faecalis</i> strain No. 52-10	Blood-agar base (Difco)
<i>Viridans streptococcus</i> strain No. 54-1	Blood-agar base (Difco)
<i>Candida albicans</i>	Nutrient agar (Difco)

RESULTS

Samples of sterile parotid saliva from fifty-six human beings were inoculated with one or more of ten different microbes. The limited supply of saliva did not allow testing of each microbe in an aliquot of each specimen; however, several salivas were used more than once as the sole source of nutrient. The results indicated that the populations of a staphylococcus, an enterococcus, a Gram-negative bacillus, a yeast, and spores and vegetative cells of a strain of *B. subtilis* var. *niger* and a strain of *B. cereus* var. *terminalis* tended to survive and multiply in the parotid saliva. Populations of a lactobacillus, a β -streptococcus, a viridans streptococcus and a

diphtheria bacillus were unable to survive and multiply. Representative growth curves from these two groups of microbes have been selected to describe the results in greater detail.

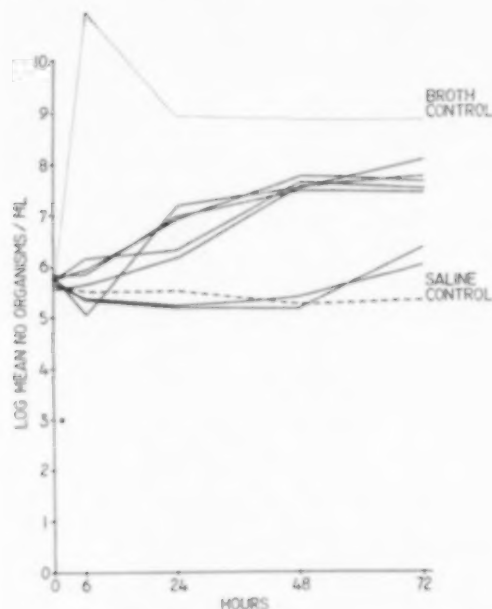


FIG. 2. Number (\log_{10}) of viable *B. subtilis* after inoculation and incubation of spores in buffered human parotid saliva (solid lines), buffered nutrient broth (broken line) and buffered isotonic saline.

The experiments with spore-forming bacilli furnished the most interesting results. The first of a series is presented graphically in Fig. 2. The inoculum was from a washed suspension of a 2 week old culture of *B. subtilis* var. *niger*. Although stained smears showed that nearly all the cells were endospores, some vegetative cells were present in each field. Since such a population of spores might be inoculated into the mouth directly or via an inanimate object, it was decided to use the suspension without heat treatment. Each curve is based on single points representing the logarithms (to the base 10) of the averages of counts from duplicate platings at each time period. The counts of viable cells arising from spore germination and cell multiplication show that in the control tube containing nutrient broth there was a rapid increase in cells from about 850,000 cells/ml at 0 hr to nearly 10^9 /ml after 6 hr of incubation. The level dropped about a hundredfold after 24 hr and remained steady through 72 hr. There was little change in the number of viable cells arising from suspension of the inoculum in the isotonic saline control.

The curves for these seven saliva samples are separable into two groups. The first group of five samples supported steady increases in cells through 48 hr of incubation. There was a further increase in cells in one saliva sample at 72 hr, but

little change occurred in the number of cells in the other samples. Even though the level of these counts at 48 and 72 hr was almost a hundredfold higher than at 0 hr, the counts were tenfold lower than those from nutrient broth at comparable periods. The major increase in cells in nutrient broth occurred during the first 6 hr whereas in the salivas the number of viable cells increased much more slowly.

TABLE 1. SUMMARY OF pH MEASUREMENTS

Microbe	Range for growth in prepared medium	Range for salivas at		Buffered saliva at		Culture medium at	
		0 hr	72 hr*	0 hr	72 hr*	0 hr	72 hr*
<i>S. aureus</i>	5.0-8.0	8.0-8.5	8.0-8.7†	7.7	7.9†	7.3	—
<i>C. albicans</i>	4.0-9.0	8.0-8.6	7.1-8.6	7.7	7.7	6.6	7.4
<i>Lactobacillus</i> sp.	5.0-8.0	7.8-8.6	8.0-8.6	7.4	7.3	5.8	4.3
<i>Str. pyogenes</i>	7.0-8.0	8.0-8.6	8.0-8.7	7.0	7.1	7.2	6.5
<i>Viridans strep.</i>	5.0-9.0	8.1-8.8	7.9-8.6	6.9	6.9	7.3	6.5
<i>Str. faecalis</i>	6.0-9.0	8.0-8.6	7.3-8.8	7.5	7.5	7.3	6.8
<i>A. cloacae</i>	5.0-8.0	8.0-8.8	7.8-8.8	7.5	7.5	7.3	7.7
<i>C. diphtheriae</i>	6.0-8.0	7.9-8.7	7.2-8.6	6.9	6.9	7.3	7.4
<i>B. subtilis</i> var. <i>niger</i>	—	—	—	7.3	7.5	6.5	7.2
<i>B. cereus</i> var. <i>terminalis</i>	—	—	—	7.6	7.3	6.6	7.5

* After incubation aerobically at 37°C.

† These measurements were made at 48 hr.

The counts from the second group of salivas were noticeably different. No great change in the number of cells occurred until after the 48 hr period, then cells began to increase at a rate roughly equivalent to the other salivas after 6 hr of incubation. Thus there was a lag in germination of spores or multiplication of vegetative cells for at least 48 hr. The pH measurements during incubation (Table 1) indicated that hydrogen ion concentration did not vary from optimal pH range of growth sufficiently to explain this effect.

A second series of experiments was planned to determine whether sporulation occurred during the growth of vegetative cells of *B. subtilis* var. *niger*. A total of four saliva samples was used. None was from a subject whose saliva was included in the preceding tests. Each sample was separated into two equal aliquots. Each aliquot received the same size inoculum from a washed suspension of spores. The inocula were heated to 60°C for 30 min before use. The duplicate sets of tubes were incubated simultaneously. After 6 hr, one tube of each duplicate was heated to 60°C for 30 min, the second duplicate was not heated. Then, both tubes were used to determine the number of remaining viable cells. It was assumed that only spores would be viable in the heated tube, but the other tube would contain spores plus vegetative cells. The results are presented in Figs. 3A and 3B. It is apparent in Fig. 3A that the spores germinated in saliva, but there was relatively little multiplication of the vegetative cells compared to the broth control. The viable counts at 24 hr are 6-7 times higher than the 0 hr counts for salivas Nos. 15 and 16, but there is essentially no increase

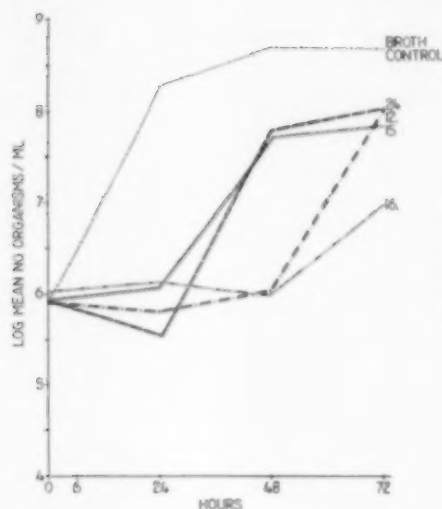


FIG. 3A. Number (\log_{10}) of viable *B. subtilis* (vegetative cells and spores) after inoculation and incubation of spores in buffered human parotid saliva and buffered nutrient broth.

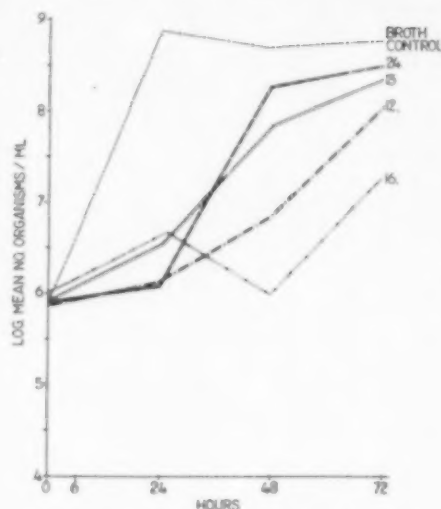


FIG. 3B. Number (\log_{10}) of viable *B. subtilis* spores after inoculation and incubation in buffered human parotid saliva and buffered nutrient broth. Tubes heated to 60°C for 30 min before plating.

in viable cells in salivas Nos. 12 and 24. After 24 hr there was marked multiplication in Nos. 15 and 24 with a slower increase in cells for No. 12. The decrease in viable cells in No. 16 is not yet accountable. All showed increases in viable cells at 72 hr.

Fig. 3B indicates the extent of sporulation in the salivas. Even though there was little multiplication of vegetative cells, sporulation occurred as evidenced by an unaltered number of viable spores during 24 hr of incubation. There would have been a decrease in the number of spores as the spores germinated, if there had been no sporulation. Note that the increase in viable spores in salivas Nos. 15 and 24 lags just behind the increase in viable vegetative and spore cells in the unheated tubes. Comparison of the numbers indicates that most of the cells in these cultures were spores. There was no increase in spores in No. 12 even though vegetative cells multiplied (see Fig. 3A) during the second day. Sporulation occurred rapidly after 48 hr of incubation. Also in No. 16 sporulation lagged behind multiplication of vegetative cells.

These results indicate that conditions of optimal growth and sporulation of *B. subtilis* var. *niger* were not favourable in these salivas during the first day of incubation. A favourable environment did not occur until after 48 hr in two of the salivas. Thus there seems to be evidence for nutritional differences between these salivas, which is similar to that observed in Fig. 2.

It was decided to determine whether similar differences between salivas obtained when a cell suspension from a strain of *B. cereus* var. *terminalis* was used as the inoculum. The results are presented in Figs. 4A and 4B. The experiment was

performed in the same way as the previous work. All samples of parotid saliva were different from those used previously except one. Sample No. 16 was an aliquot from the same batch of saliva that was used in the work with *B. subtilis* spores.

Germination and cell multiplication occurred in saliva samples during 24 hr of incubation as seen in Fig. 4A. The total number of viable cells was higher in salivas Nos. 16, 19, 23₂ and 29 than No. 17. The level of the former counts were almost equal to nutrient broth. The counts for salivas Nos. 16, 23₂ and 29 increased slightly during the next 48 hr of incubation, but these increases were small compared to the previous 24 hr. There was a temporary reduction in viable cells in No. 19 after 24 hr, but an increase to the previous level after 72 hr. No data are available to explain these changes. Saliva No. 17 did not support further multiplication of cells until after 48 hr of incubation. The total number of viable cells was nearly equal to the other salivas at 72 hr.

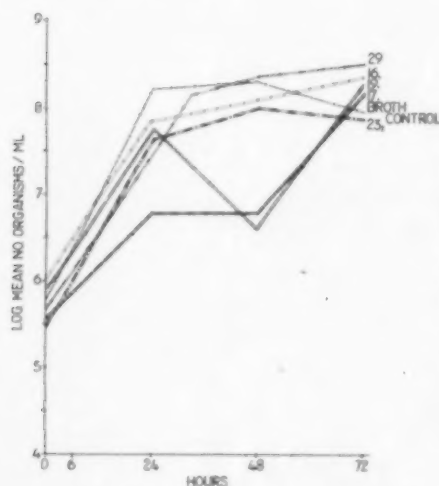


FIG. 4A. Number (\log_{10}) of viable *B. cereus* (vegetative cells and spores) after inoculation and incubation of spores in buffered human saliva and buffered nutrient broth.

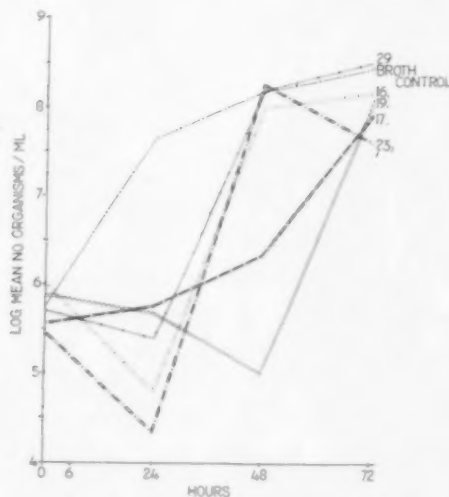


FIG. 4B. Number (\log_{10}) of viable *B. cereus* spores after inoculation and incubation of spores in buffered saliva and buffered nutrient broth. Tubes heated to 60°C for 30 min before plating.

The results presented in Fig. 4B are from tubes which had been heated to 60°C for 30 min before culturing. Sporulation occurred in nutrient broth during the first 24 hr and a comparison of the counts with those in Fig. 4A indicates that most of the viable cells in the broth cultures were spores. There was a progressive decrease in the number of viable spores in salivas Nos. 16 and 23₂ indicating germination of spores and multiplication of vegetative cells without equivalent sporulation during the first 24 hr of incubation. However, after 48 hr there was a major increase in sporulation suggesting that many cells sporulated at one time. Although there was an increase in vegetative cells in the remaining salivas, conditions were not suitable

for a high rate of sporulation during the first 24 hr period. Sporulation occurred rapidly in saliva No. 29 during the next 24 hr so that the numbers of viable spores in salivas Nos. 16, 23₂ and 29 are the same as for nutrient broth and remained almost the same for the rest of the observation period. There was a gradual increase in the number of viable spores in saliva No. 17 during 48 hr of incubation, then a major increase occurred during the last 24 hr. The final number was nearly the same as the number of spores in the other salivas. A longer delay in sporulation occurred in saliva No. 19 but approximately the same number of viable spores, as in other saliva tubes, was present after 72 hr of incubation. The increase in viable spores seemed to have the same slope as in salivas Nos. 16 and 23₂. These data indicate that the nutrients for sporulation by this strain of *B. cereus* are somewhat more critical than for sporulation by the *B. subtilis* strain.

The results of experiments with the other bacteria which tended to grow in parotid saliva did not show as clear cut differences between salivas as observed with the spore-forming bacilli. Thus if the bacterium was observed to remain viable or multiply during the incubation period in one saliva sample, usually the same type of result was observed in the salivas from all of the other subjects. However, several observations are worthy of description here. The cells of the strain of *Staphylococcus aureus* were particularly susceptible to changes in the hydrogen ion concentration. As the pH increased in the saliva, as is seen in Table 1, the staphylococci tended to die. When the saliva was buffered the bacteria tended to remain viable for the entire incubation period without an appreciable change in total numbers. The cells of the

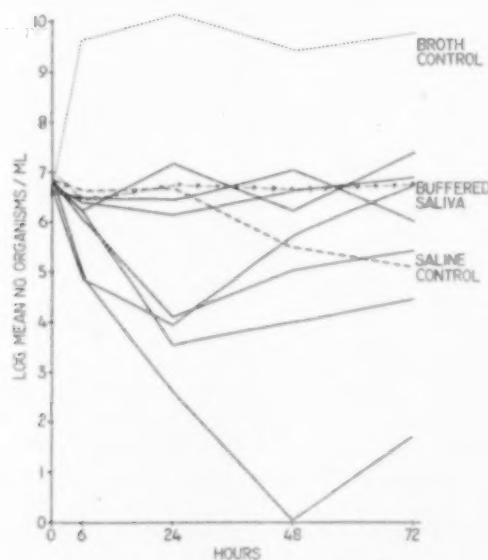


FIG. 5. Number (\log_{10}) of viable *A. cloacae* after inoculation and incubation in human parotid saliva (solid lines), buffered human parotid saliva (arrows), brain heart infusion broth control (broken lines), and isotonic saline.

strain of *Candida albicans* remained viable in the saliva with no significant change in total number for the entire 72 hr incubation period, the response was the same in all salivas regardless of buffering.

Fig. 5 presents the results of incubating the cells of a strain of *Aerobacter cloacae* in seven separate samples of parotid saliva. Each solid black line represents a different saliva sample, but each is not identified. The points were determined by the average of viable counts at each time period. Note that the organisms tend to survive in three of the salivas with sporadic indications of cell multiplication. These increases are small compared to the broth control. There were three salivas in which the viable counts decreased progressively through 24 hr, but then began to show cell multiplication; again very much less than in the broth control. The cells in the last saliva died progressively over the 48 hr period and then began to multiply. It is not clear, in these last four salivas, whether the dead cells furnished nutrients for the viable cells; or whether a variant or a mutant developed.

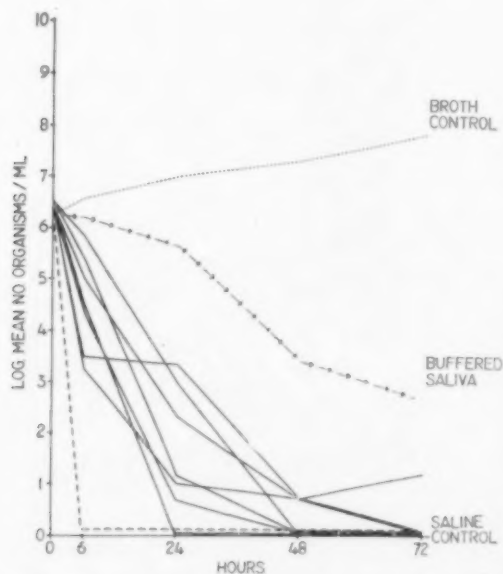


FIG. 6. Number (\log_{10}) of viable viridans streptococci after inoculation and incubation in human parotid saliva (solid lines), buffered human parotid saliva (arrows), brain heart infusion broth (broken line), and isotonic saline.

The patterns of decreasing viability among the bacteria which did not survive in parotid salivas were essentially the same regardless of the saliva sample. Fig. 6 presents the curves from salivas inoculated with cells from an oral strain of a viridans streptococcus. Note that the rapid and progressive loss of viability is almost the same as in the isotonic saline control. There appears to be some protection when the saliva

is buffered, but there is still a loss of viability. Data on the survival of diphtheria bacilli and pyogenic streptococci are not presented because of almost identical, if not more rapid, death of the cells compared with the viridans streptococci. The results using cells from a heterofermentative lactobacillus, presented in Fig. 7, are very similar. There are two salivas, however, which showed a sudden change in the number of cells; it can only be assumed that certain cells could synthesize or utilize nutrients that accumulated in the saliva and thus began to multiply. It is observed that multiplication lasted only for 24 hr, then progressive death of cells occurred

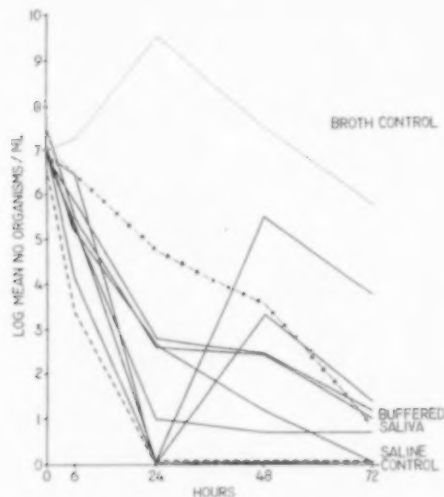


FIG. 7. Number (\log_{10}) of viable lactobacilli after inoculation and incubation in human parotid saliva (solid lines), buffered human parotid saliva (arrows), SL broth (broken line), and isotonic saline.

again. We were not prepared to attempt isolation and preservation of these cells, and thus these experiments will bear repeating. The sudden appearance, in an apparently dying population, of cells which rapidly increase in number suggests adaptation, variation or mutation. This assumption is strengthened by almost identical changes in these two separate saliva samples.

DISCUSSION

The oral flora exists in an "open system" (VON BERTALANFFY, 1950) and might be considered a natural "chemostat". A better understanding of its composition, products and interactions can be attained only by study of its several characteristics separately and together. A series of studies in our laboratories have been directed toward these goals (WILLIAMS, EICKENBERG and FLOREY, 1953; WILLIAMS, OSHTRY

and BAUMBACH, 1956; and ZELDOW, 1955). The importance of salivary secretions in oral ecology has been recognized from the outset, but only recently have procedures been described which would allow study. ZELDOW (1955) reported the application of techniques to the collection of human parotid saliva which resulted in a marked reduction in viable microbes in the fluid. Modification of these techniques allowed collection of parotid saliva samples which were free of cultivable microbes. This development suggested a study using parotid saliva as the sole source of nutrients for oral microbes (WILLIAMS, 1953).

Since saliva, under natural conditions, exists as a film on the surfaces of mucous membranes, it was considered desirable to study the effects of the parotid saliva while in a film on glass. HEUKELEKIAN and HELLER (1940) indicated that the growth of bacteria in dilute culture media could be accelerated by increasing the surface-volume ratio with sterile sand or glass beads. They also found that if air was bubbled through the medium, higher counts resulted. The roller tubes, used in this work, were designed to increase the surface-volume ratio of saliva and to agitate the saliva. Preliminary studies with nutritionally adequate medium indicated that the counts from the roller tubes were consistently higher than counts from identical tubes, with the same amount of medium, held in the upright position. Acceleration of growth was only observed with the Bacilli. These findings suggested that roller tubes might provide a better environment and opportunity for microbial growth in saliva than tubes in the conventional position.

In general, all human parotid salivas were less than optimal media for all microbes. However, the growth of the Bacilli tended to be supported better than most other microbes. There were only two salivas which did not support germination or growth of vegetative cells. These results indicate that the washing of the spore suspensions was sufficient to remove residual nutrients from the spores (HALVORSON and CHURCH, 1957) otherwise all cultures would have shown the same degree of germination. The requirements for germination of *B. subtilis* and *B. cereus* spores are different in that *B. subtilis* spores will germinate in the presence of a larger variety of amino acids (HALVORSON and CHURCH, 1957). In parotid saliva the spores of *B. cereus* germinated more rapidly than did the spores of *B. subtilis* (see Fig. 3A and 4A). The significance of this observation cannot be completely understood until the results of assays of amino acids and of other growth stimulants in parotid salivas have been completed. In any event the germination of bacterial spores indicates that parotid saliva contains at least a nitrogen source, a carbon source and a precursor of nucleic acids (HALVORSON and CHURCH, 1957).

The greatest nutritional differences between salivas was noted when sporulation of the Bacilli was used as the criterion. Sporulation in the cells of the strain of *B. cereus* seemed to be slightly more sensitive to nutrients in the medium than the cells of *B. subtilis*. However, the use of sporulation to assay human parotid saliva for several chemical substances seems possible with both kinds of sporulating bacteria because of their differences in nutritional requirements (ORDAL, 1957). Recent study of the minerals in human parotid saliva (JACKSON and SECORD, 1959) has indicated that manganese (Mn^{2+}), an important mineral for sporulation (CURRAN, 1957), was

present in nineteen separate parotid salivas in concentrations ranging from 0.4 to 0.9 mequiv./l. (arithmetic mean was 0.6 mequiv./l.). The lower levels in these salivas are near the critical concentrations needed for sporulation. The variation in manganese content may explain the observed differences in sporulation in parotid saliva. It remains to be seen whether the presence or absence of specific nutrients in parotid saliva from particular human beings is a consistent finding.

There is evidence from these experiments that the low hydrogen ion concentrations which develop immediately after collection of parotid saliva interfere with the survival of several kinds of bacteria. Addition of phosphate buffer altered the saliva sufficiently for survival to be prolonged for cells of lactobacilli, β -streptococci and diphtheria bacilli. Saliva was collected under oil in some of the preliminary studies but it interfered with the culture work. It was found that the pH of the saliva collected and maintained under oil remained constant for only a few hours. If the saliva was not refrigerated, the pH tended to rise whether microbes could be isolated or not. Thus, the loss of carbon dioxide (HODGE and LEUNG, 1950; LEUNG, 1951) did not account entirely for the pH changes. It is suspected that salivary proteases similar to those reported by CHAUNCEY, JOHNSON and LISANTI (1954) are responsible for early changes, although a specific test of this possibility was not attempted.

The detrimental effects of isotonic saline on microbes washed in distilled water was greatest with the streptococci. It is believed that this treatment altered the osmotic relationships of the cells, increasing their sensitivity (MITCHELL, 1951). This effect was reduced somewhat when the microbes were suspended in parotid saliva, or gelatin to a concentration of 0.1 per cent was added to the isotonic saline solution.

The data obtained in these studies are not sufficient to answer all questions concerning the role of human parotid saliva in oral ecology. They do indicate that, under the conditions of these experiments, saliva from certain human beings affects the same microbial strains in much the same way. There are obvious major differences such as the effects on spore suspensions of *B. subtilis* and *B. cereus*. In view of opinions that the oral flora is reasonably characteristic for different human beings (WILLIAMS *et al.* 1953; KRAUS and GASTON, 1956) it would appear that parotid saliva may contribute to the control of the oral flora. The best examples were the variable delay of sporulation and the several effects on cells of an *Aerobacter* and lactobacillus.

These and further studies may also lead to a better understanding and perhaps control of postoperative parotitis which may follow general or oral surgery (APPLETON, 1950). Although microbes may gain access to the glands haematogenously, the findings reported here indicate that if microbes gain access through Stenson's duct several kinds could metabolize in the static fluid, initiating an "ascending infection". The reduction in flow rate may occur from use of drugs prior to surgery or the patients' response to the anaesthetic or surgery.

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THE RELATIONSHIP BETWEEN LACTOBACILLUS COUNTS, SNYDER TESTS AND THE SUBSEQUENT INCIDENCE OF DENTAL CARIES

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Abstract—A significant association was found between lactobacillus counts, Snyder tests and the number of newly decayed tooth surfaces in 584 patients. The results from each test were similar. The value of a scoring system for recording the results of each test is questioned and it is concluded that a simplified system of recording a positive or negative result is all that is required.

Tomato juice-sodium azide agar was the medium used for growing lactobacilli. Preliminary biochemical tests suggest that this medium is selective for lactobacilli.

Lactobacillus counts and Snyder tests were more reliable for predicting the incidence of caries in young children aged 3-13 years than in older patients aged 12-31 years. Neither test is reliable for predicting the incidence of caries over a period of time exceeding one year.

The value of the tests as research and health-education tools is discussed.

INTRODUCTION

The lactobacillus count was introduced as a laboratory test for evaluating caries activity by HADLEY in 1933. Seven years later SNYDER (1940) introduced the simpler colorimetric test for the same purpose.

Since then both tests have been used extensively as research and diagnostic tools. In some studies the results of the tests have been compared with the results of clinical examinations which were completed at the same time as the tests (i.e. the prevalence of dental caries). Others have compared the results of the tests with the number of new lesions which occur in a specified interval of time after the tests were done (i.e. the incidence of dental caries). In both cases the results have been contradictory (SLACK and MARTIN, 1956).

In our opinion, the practical value of these susceptibility tests will depend upon their reliability as a method of predicting the number of lesions in a specified time after the tests are done. Alternatively, if the results of the tests depend solely upon the number of active lesions present at the time of examination, as Cox (1952) implies, then the tests would only be worth doing if they made it possible to define the rate at which the existing lesions would progress.

Since we were more interested in the first of these possibilities this study was organized in an attempt to find answers to the following questions: first, is there a relationship between the numbers of lactobacilli in saliva, the results of Snyder tests and the number of newly decayed tooth surfaces which develop in various intervals of time after the tests are done? Secondly, if these factors are related, how reliable are the tests for predicting the subsequent number of new lesions?

MATERIAL

Five hundred and eighty-four patients divided into two groups took part in this study. The first group consisted of 322 patients aged 12-31 years who volunteered to participate in a study of the effect of an ammoniated dentifrice on the incidence of dental caries during a period of nine months (DAVIES and KING, 1951). The data from this group were included in this study because the results from the experiment with the ammoniated dentifrice revealed: first, that the number of new lesions was the same in both the experimental and control groups and secondly, that at both the beginning and the end of the experiment there was no significant difference between the lactobacillus counts of the experimental and control groups. The second group consisted of 262 children aged 3-13 years who attended the Department of Children's Dentistry for routine dental treatment.

METHODS

Examination. All participants were given a careful clinical examination in a dental chair using plane mouth mirrors and sharp explorers. Compressed air was used to dry each tooth before it was examined. Lighting consisted of daylight supplemented with fluorescent lighting.

The first group of 322 patients was examined by DAVIES and KING. The second group of children was examined by final year dental students but each examination was checked by DAVIES or KING.

Bite-wing radiographs were taken for all patients except those whose posterior teeth were not in contact. A viewing box and magnifying glass were used for the interpretation of the radiographs, all of which were examined by DAVIES.

Collection of saliva. Specimens of saliva were collected in sterile sample bottles from each patient on two or three successive mornings after the initial clinical examination. Salivary flow was stimulated by chewing paraffin wax and all samples were collected before the patients got out of bed in the morning. For the first group of 322 patients, aged 12-31 years, the time of collection was standardized at 5 min. For the second group of children, aged 3-13 years, the time of collection ranged from 5 to 20 min.

Saliva samples were delivered to the Dental School on the morning of collection and were kept in a refrigerator until the tests could be done later on the day of collection. Each sample of saliva was submitted to a lactobacillus count and a Snyder test.

Snyder test. Three drops (approximately 0.2 ml) of each well-shaken sample of saliva were added from a graduated 1 ml pipette to 5 ml of Snyder's medium (Difco) at approximately 45°C. The inoculated medium was cooled by placing the tube in cold water, after which it was placed in an incubator at 37°C. Each tube was examined for colour change after 24, 36, 48, and 72 hr. A positive reaction was recorded when green was no longer the predominant colour.

Lactobacillus count. Each sample of well-shaken saliva was diluted by adding 1 ml of the sample to 4 ml of beef extract broth at pH 5.0. Then 0.1 ml of the diluted

sample was transferred to a plate of tomato-juice agar (SHROFF and MADDEN, 1948) to which 1:10,000 sodium azide (DIAMOND, 1950) had been added to inhibit the growth of yeasts. The pH of the tomato-juice agar was 5.0. The sample was distributed evenly over the surface of the plate with a glass spreader. All inoculated plates were incubated at 37°C for 72 hr before the colonies were counted.

Because SHROFF and MADDEN's medium is different from that used by HADLEY (1933) it was decided that the lactobacillus counts would have to be scored according to a system different from that used by HADLEY. Preliminary work with SHROFF and MADDEN's medium suggested that the critical number of lactobacilli per millilitre of saliva was 10,000 for young children and 40,000 for adults and adolescents. Children with lactobacillus counts greater than 10,000, and adolescents and adults with counts greater than 40,000 had significantly more decayed tooth surfaces than did patients of the same age with counts below 10,000 and 40,000 respectively. This hypothesis was confirmed statistically by plotting the cumulative percentage of carious tooth surfaces against the log of the lactobacillus count multiplied by 10^{-3} . Since a dilution factor of fifty was used, one colony on the medium represents fifty organisms in the sample. Accordingly, the scoring systems adopted in this study were as follows: for patients aged 12-31 years—nil, 50-40,000 and 40,050 or more; for patients aged 3-13 years—nil, 50-10,000 and 10,050 or more.

Identification of colonies growing on tomato juice-sodium azide agar. Fifty colonies, from seven different specimens, were picked off the tomato juice-sodium azide agar, subcultured in tomato-juice broth (DAVIS, BISSET and HALE, 1955) and examined according to HAYWARD's modification (HAYWARD and DAVIS, 1956) of the biochemical tests of DAVIS (1955).

Statistical analysis. Since the numbers of patients in some of the groups were small, the significance of the differences in mean values was assessed by Student's "t" test.

RESULTS

Association of lactobacillus counts and Snyder test results. The distribution of 322 patients aged 12-31 years according to the results of the lactobacillus counts and Snyder tests is shown in Table 1. The application of X^2 test to these data reveals a

TABLE 1. DISTRIBUTION OF 322 PATIENTS AGED 12-31 YEARS ACCORDING TO THE RESULTS OF LACTOBACILLUS COUNTS AND SNYDER TESTS

Lactobacillus count per millilitre of saliva	No. of patients with stated Snyder test result			Total no. of patients
	Negative	Positive after 37-72 hr	Positive after 36 hr or less	
Nil	17	12	0	29
50-40,000	8	32	34	74
40,050 or more	3	43	173	219
Total No. of patients	28	87	207	322

significant association between the results of the two tests ($X^2=176.78$, degrees of freedom=4, $P=<0.001$).

The distribution of 262 children aged 3-13 according to the results of the lactobacillus counts and Snyder tests is shown in Table 2. The application of X^2 test to these data also reveals a significant association between the results of the two tests ($X^2=210.75$, degrees of freedom=4, $P=<0.001$).

TABLE 2. DISTRIBUTION OF 262 PATIENTS AGED 3-13 YEARS ACCORDING TO THE RESULTS OF LACTOBACILLUS COUNTS AND SNYDER TESTS

Lactobacillus count per millilitre of saliva	No. of patients with stated Snyder test result			Total no. of patients
	Negative	Positive after 37-72 hr	Positive after 36 hr or less	
Nil	140	21	2	163
50-10,000	5	37	11	53
10,050 or more	0	16	30	46
Total No. of patients	145	74	43	262

Association of lactobacillus counts and the incidence of dental caries. The general trend of these results, which are set out in Tables 3 and 4, shows that, regardless of the length of time between examinations, the incidence of dental caries is highest in patients with high lactobacillus counts, intermediate in patients with low lactobacillus counts and least in patients with negative counts. However, in both the group of children aged 3-13 years and in the group of patients aged 12-31 years the number of newly decayed tooth surfaces in patients with high counts was not significantly different from that in patients with low counts. Because of this finding the data was reorganized to determine the significance of the differences in the number of new lesions between patients with positive and negative lactobacillus counts. The data pertaining to children aged 3-13 years are set out in Table 5, and the data pertaining to the patients aged 12-31 years are set out in Table 6.

These results demonstrate that, in both groups, patients with positive lactobacillus counts developed a significantly higher number of new lesions in 12 months or less than patients with negative counts. Young children with positive lactobacillus counts who were examined 13 months or more after the counts were made also developed more new lesions than children with negative counts who were examined 13 months or more after the counts were made, but the difference was not significant.

Association of Snyder test results and the incidence of dental caries. The data for the 3-13 year old children are set out in Table 7 and those for the 12-31 year old patients are set out in Table 8. Although the general trend of these results suggests that the incidence of caries is related to the results of Snyder tests the number of children in some of the groups is small. For both groups patients with strongly positive Snyder tests had a higher number of new lesions in 12 months or less than

did patients with negative Snyder tests. However, in neither group were the numbers of new lesions significantly higher in the patients with strongly positive Snyder tests than in those with weakly positive Snyder tests. Because of this the data were reorganized to determine the significance of differences in the incidence of caries between patients with positive and negative Snyder tests (Tables 9 and 10).

TABLE 3. DIFFERENCES BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 3-13 YEAR OLD CHILDREN WITH DIFFERENT LACTOBACILLUS COUNTS WHO WERE RE-EXAMINED 6 MONTHS OR LESS, 7-12 MONTHS AND 13 MONTHS OR MORE AFTER THE LACTOBACILLUS COUNTS WERE MADE

Lactobacillus counts per millilitre of saliva	No. of children	Mean no. newly decayed tooth surfaces	Standard deviation	Difference between means	Standard error of difference	"t"	P
6 months or less between examinations							
Nil	52	1.50	2.41	1.57	0.86	1.82	N.S.
50-10,000	14	3.07	2.97				
Nil	52	1.50	2.41	2.90	1.16	2.50	0.02
10,050 or more	10	4.40	3.52				
50-10,000	14	3.07	2.97	1.33	1.36	0.98	N.S.
10,050 or more	10	4.40	3.52				
7-12 months between examinations							
Nil	92	1.98	2.71	2.14	0.68	3.15	0.01
50-10,000	33	4.12	3.57				
Nil	92	1.98	2.71	2.26	0.64	3.53	0.01
10,050 or more	25	4.24	2.88				
50-10,000	33	4.12	3.57	0.12	0.85	0.14	N.S.
10,050 or more	25	4.24	2.88				
13 months or more between examinations							
Nil	19	3.16	2.95	0.34	1.63	0.21	N.S.
50-10,000	6	3.50	3.63				
Nil	19	3.16	2.95	1.38	1.26	1.09	N.S.
10,050 or more	11	4.54	3.52				
50-10,000	6	3.50	3.63	1.04	1.82	0.57	N.S.
10,050 or more	11	4.54	3.52				

The results are similar to those obtained for positive and negative lactobacillus counts. Patients with positive Snyder tests developed a significantly higher number of new lesions in 12 months or less than patients with negative Snyder tests. Although young children with positive Snyder tests also developed more new lesions 13 months or more after the tests were done than children of the same age with negative counts, the difference was not significant.

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TABLE 4. SIGNIFICANCE OF THE DIFFERENCES IN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 12-31 YEAR OLD PATIENTS WITH DIFFERENT LACTOBACILLUS COUNTS WHO WERE RE-EXAMINED 9 MONTHS AFTER THE COUNTS WERE MADE

Lactobacillus counts per millilitre of saliva	No. of patients	Mean no. newly decayed tooth surfaces per patient	Standard deviation	Difference between means	Standard error of difference	"t"	P
Nil	29	4.03	4.21	2.32	0.95	2.44	.01
50-40,000	74	6.35	4.70				
Nil	29	4.03	4.21	2.65	0.84	3.15	.01
40,050 or more	219	6.68	4.78				
50-40,000	74	6.35	4.70	0.33	0.64	0.52	N.S.
40,050 or more	219	6.68	4.78				

The suitability of lactobacillus counts and Snyder tests for predicting the incidence of dental caries. The data pertaining to the children aged 3-13 years showed that there were no significant differences in the numbers of newly decayed tooth surfaces, 13 months or more after the initial examinations, between children with positive and negative lactobacillus counts nor between children with positive and negative Snyder tests. It may be concluded, therefore, that neither saliva test can be used to predict the incidence of caries over a period of time exceeding one year. There were, however, significant differences in the incidence of caries over a period of less than a year between children and young adults with positive and negative saliva tests.

TABLE 5. SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 3-13 YEAR OLD CHILDREN WITH POSITIVE AND NEGATIVE LACTOBACILLUS COUNTS WHO WERE RE-EXAMINED 6 MONTHS OR LESS, 7-12 MONTHS AND 13 MONTHS OR MORE AFTER THE LACTOBACILLUS COUNTS WERE DONE

Lactobacillus counts per millilitre of saliva	No. of children	Mean no. newly decayed tooth surfaces per child	Standard deviation	Difference between means	Standard error of difference	"t"	P
6 months or less between examinations							
Negative	52	1.50	2.41	2.12	0.71	2.98	0.01
Positive	24	3.62	3.10				
7-12 months between examinations							
Negative	92	1.98	2.71	2.19	0.50	4.38	0.01
Positive	58	4.17	3.22				
13 months or more between examinations							
Negative	19	3.16	2.95	1.02	1.06	0.96	N.S.
Positive	17	4.18	3.33				

To determine the practical value of each test as a method of forecasting the number of new lesions, calculations were made of the percentage of 3-13 year old children with negative and positive lactobacillus counts and Snyder tests who developed 0-2, 3-5, and 6 or more newly decayed tooth surfaces in 6 months or less and in 7-12 months. For patients aged 12-31 years calculations were made of the percentage of patients with negative and positive lactobacillus counts and Snyder tests who developed 0-5, 6-11, and 12 or more newly decayed tooth surfaces in 9 months. The selection of the class intervals for the number of newly decayed tooth surfaces was based upon the mean number of new lesions per person which developed in 6 months or less in the seventy-six children aged 3-13 years, in 7-12 months in the 150 children aged 3-13 years, and in 9 months in the 322 patients aged 12-31 years. The means for these groups were 2.17, 2.83 and 6.36 newly decayed tooth surfaces respectively. (The respective standard deviations were 2.39, 3.09 and 4.78). The results of these calculations are set out in Table 11.

TABLE 6. SIGNIFICANCE OF THE DIFFERENCE BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 12-31 YEAR OLD PATIENTS WITH POSITIVE AND NEGATIVE LACTOBACILLUS COUNTS WHO WERE RE-EXAMINED 9 MONTHS AFTER THE LACTOBACILLUS COUNTS WERE MADE

Lactobacillus counts per millilitre of saliva	No. of patients	Mean no. newly decayed tooth surfaces per patient	Standard deviation	Difference between means	Standard error of difference	"t"	P
Negative	29	4.03	4.21	2.56	0.83	3.08	0.01
Positive	293	6.59	4.77				

In simple terms these results mean that approximately 71-79 per cent of 3-13 year old children with negative Snyder tests or negative lactobacillus counts developed less than three newly decayed tooth surfaces in 12 months.

Thus when saliva tests were carried out according to the methods adopted in this study it could be predicted that the chances of young children with negative lactobacillus counts or negative Snyder tests developing less than three carious lesions in the following 12 months are about three to one, whereas young children with positive saliva tests have almost a three to one chance of developing three or more new carious lesions in the following 12 months.

The probability is lower for patients aged 12-31 years. In this case 36 per cent of those with negative Snyder tests and 31 per cent of those with negative lactobacillus counts developed six or more newly decayed tooth surfaces in the following 9 months; whereas 53 per cent of those with positive Snyder tests and 54 per cent of those with positive lactobacillus counts developed six or more new lesions in the same period of time.

Identification of colonies growing on tomato juice-sodium azide agar. Of the fifty colonies examined, eleven were Type 1 round, cream in colour, hemispherical, and about 1 mm in diameter; two were Type 2 about 1 mm in diameter, round and white

with a raised and cream centre; and thirty-seven were Type 3 fine clear colonies ranging from 0.05–0.2 mm in diameter. The morphological appearances of the three types after incubation for 24 hr in tomato-juice broth were as follows.

Type 1. Gram-positive, short, squat bacilli arranged singly or in pairs.

Type 2. Gram-positive, short, regular bacilli arranged singly or in pairs.

Type 3. Gram-positive rods about 3μ in length, arranged regularly in pairs and short chains.

The results of the biochemical tests are set out in Table 12. All the strains were non-motile, Gram-positive bacilli which did not produce catalase and which did not

TABLE 7. SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 3–13 YEAR OLD CHILDREN WITH DIFFERENT SNYDER TEST RESULTS WHO WERE RE-EXAMINED 6 MONTHS OR LESS, 7–12 MONTHS AND 13 MONTHS OR MORE AFTER THE TESTS WERE MADE

Snyder test results*	No. of children	Mean no. newly decayed tooth surfaces	Standard deviation	Difference between means	Standard error of difference	"t"	P
6 months or less between examinations							
Nil	47	1.70	1.84				
+	21	2.14	2.58	0.44	0.62	0.71	N.S.
Nil	47	1.70	1.84				
++	8	5.00	3.68	3.30	1.33	2.48	0.02
+	21	2.14	2.58				
++	8	5.00	3.68	2.86	1.41	2.02	N.S.
7–12 months between examinations							
Nil	81	1.53	2.19				
+	40	4.55	3.57	3.02	0.61	4.95	.01
Nil	81	1.53	2.19				
++	29	4.07	3.01	2.54	0.61	4.16	.01
+	40	4.55	3.57				
++	29	4.07	3.01	0.56	0.79	0.71	N.S.
13 months or more between examinations							
Nil	17	3.12	2.99				
+	13	4.31	3.52	1.19	1.22	0.97	N.S.
Nil	17	3.12	2.99				
++	6	3.67	3.31	0.55	1.53	0.36	N.S.
+	13	4.31	3.52				
++	6	3.67	3.31	0.64	1.67	0.38	N.S.

* Nil = negative Snyder test result.

 + = weakly positive Snyder test result.

 ++ = strongly positive Snyder test result.

TABLE 8. SIGNIFICANCE OF THE DIFFERENCES IN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 12-31 YEAR OLD PATIENTS WITH DIFFERENT SNYDER TEST RESULTS WHO WERE RE-EXAMINED 9 MONTHS AFTER THE TESTS WERE MADE

Snyder test results*	No. of patients	Mean no. newly decayed tooth surfaces per patient	Standard deviation	Difference between means	Standard error of difference	"t"	P
Nil	28	4.43	4.21				
+	87	6.10	4.12	1.67	0.90	1.86	N.S.
Nil	28	4.43	4.21				
++	207	6.73	5.05	2.30	0.86	2.67	0.01
+	87	6.10	4.12				
++	207	6.73	5.05	0.63	0.56	1.12	N.S.

- * Nil = negative Snyder test result.
 + = weakly positive Snyder test result.
 ++ = strongly positive Snyder test result.

reduce nitrate. Thirty-eight of the strains gave the reaction of *Lactobacillus casei* but since no attempt was made to get strain purity by alternate growth in broth and agar, exact identification is not possible. This work is now being done and will be the subject of a later report.

It appears that under the conditions of this experiment tomato juice-sodium azide agar is selective for lactobacilli. Additional tests have shown that streptococci of the

TABLE 9. SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 3-13 YEAR OLD CHILDREN WITH POSITIVE AND NEGATIVE SNYDER TEST RESULTS WHO WERE RE-EXAMINED 6 MONTHS OR LESS, 7-12 MONTHS AND 13 MONTHS OR MORE AFTER THE SNYDER TESTS WERE DONE

Snyder test results	No. of children	Mean no. newly decayed tooth surfaces per child	Standard deviation	Difference between means	Standard error of difference	"t"	P
6 months or less between examinations							
Negative	47	1.70	1.84				
Positive	29	2.93	3.02	1.23	0.62	1.98	0.05*
7-12 months between examinations							
Negative	81	1.53	2.19				
Positive	69	4.35	3.30	2.82	0.47	6.00	0.001
13 months or more between examinations							
Negative	17	3.12	2.99				
Positive	19	4.11	3.25	0.99	1.03	0.96	N.S.

* (Borderline)

viridans type would not grow on the tomato juice-sodium azide agar on initial isolation. However, they did grow on this medium when they were first isolated on blood agar and sub-cultured in "Difco" dextrose broth at pH 7.2.

TABLE 10. SIGNIFICANCE OF THE DIFFERENCE BETWEEN THE INCIDENCE OF NEWLY DECAYED TOOTH SURFACES IN 12-31 YEAR OLD PATIENTS WITH POSITIVE AND NEGATIVE SNYDER TESTS WHO WERE RE-EXAMINED 9 MONTHS AFTER THE TESTS WERE DONE

Snyder test results	No. of patients	Mean no. newly decayed tooth surfaces per patient	Standard deviation	Difference between means	Standard error of difference	"t"	P
Negative	28	4.43	4.21	2.12	0.84	2.52	0.02
Positive	294	6.55	4.79				

TABLE 11. PERCENTAGE OF PATIENTS WITH POSITIVE AND NEGATIVE LACTOBACILLUS COUNTS AND SNYDER TESTS WHO DEVELOPED STATED NUMBER OF NEWLY DECAYED TOOTH SURFACES IN VARYING LENGTHS OF TIME

Age group in years	Length of time between examinations	No. of newly decayed tooth surfaces	Percentage of patients who developed stated no. of newly decayed tooth surfaces			
			Lactobacillus count		Snyder test	
			Negative	Positive	Negative	Positive
3-13	6 months or less	0-2	79	38	74	52
		3-5	15	33	17	27
		6 or more	6	29	9	21
3-13	7-12 months	0-2	71	36	75	36
		3-5	12	34	14	29
		6 or more	17	30	11	35
12-31	9 months	0-5	69	46	64	47
		6-11	24	27	29	26
		12 or more	7	27	7	27

DISCUSSION

The results from the 584 patients who took part in this study establish that there is a significant association between both lactobacillus counts and Snyder tests and the subsequent incidence of caries. However, the increase in the number of new lesions was not significantly higher in patients with high lactobacillus counts than in those with low counts. Nor was the number of new lesions higher in patients with strongly positive Snyder tests (++) than in patients with weakly positive Snyder tests (+). We interpret these results to mean that, from a diagnostic point of view, a positive count or a positive Snyder test is just as valuable as a more detailed count

of the actual numbers of lactobacilli in a millilitre of saliva or the length of time which elapses before the indicator in Snyder's medium changes from green to yellow.

The results of this study suggest that both saliva tests can be used to predict whether or not a patient will develop more or less new lesions in the following 12 months than the average for his age. This prediction would appear to be more reliable for young children than young adults. This may be related to the fact that a negative saliva test is more often associated with low caries activity than a positive saliva test is associated with high caries activity. In this study 55 per cent of the children aged 3-13 years had a negative Snyder test and 62 per cent of the same children had a negative lactobacillus count, whereas in the 13-41 year age group only 9 per cent had negative saliva tests.

TABLE 12. DIFFERENTIAL CHARACTERISTICS OF 50 LACTOBACILLUS COLONIES ISOLATED FROM TOMATO JUICE-SODIUM AZIDE AGAR INOCULATED WITH SALIVA

Colony morphology	No. of colonies giving identical responses in biochemical tests	Results of biochemical tests									
		Gas from glucose	Arginine	Hippurate	Aesculin	Arabinose	Raffinose	Mannite	Salicin	Growth at 15°C	Growth at 45°C
Type 3	10	-	-	+	+	-	-	+	+	+	-
Type 1	5	-	-	+	+	-	-	+	+	+	-
Type 3	20	-	-	+	+	-	-	+	+	+	+
Type 1	1	-	-	+	+	-	-	+	+	+	+
Type 3	5	-	-	-	+	-	-	+	+	+	+
Type 1	1	-	-	-	+	-	-	+	+	+	+
Type 3	1	-	+	+	+	+	+	+	+	+	+
Type 3	1	-	+	+	+	+	-	+	+	+	+
Type 1	2	-	-	+	+	-	+	+	+	+	-
Type 1	1	±	+	-	-	-	+	+	-	+	-
Type 1	1	-	-	-	+	+	+	+	+	+	-
Type 2	2	-	-	-	-	-	+	+	-	+	-

It could be argued that the reliability of these tests to predict the incidence of caries is too low for the procedure to be of any value as a clinical tool. The results of this study make it clear, however, that the tests have two other important uses. First, the control and experimental groups in a clinical trial of any agent for preventing

dental caries should have equal numbers of patients with positive and negative saliva tests. Secondly, the tests may be used as an aid to dental health education so that emphasis can be placed on the need for the patients with positive Snyder tests or positive lactobacillus counts to adopt a strict programme of prevention.

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A COMPARISON OF THE INCREMENT OF FLUORIDE IN ENAMEL AND THE REDUCTION IN DENTAL CARIES RESULTING FROM TOPICAL FLUORIDE APPLICATIONS*

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Abstract—Deciduous teeth which exfoliated during and after a topical fluoride application study were collected and subjected to fluoride analysis. In the study one-half of the dentition of 107 children was treated four times with 2% NaF. The untreated teeth and the teeth of a similar group of children receiving no treatment served as controls. The caries reductions after 1, 2 and 3 years were 19, 14 and 7.5 per cent when based on a comparison of treated and untreated teeth in the same mouth, and 34, 23 and 12 per cent when teeth of untreated children were used as controls. Analysis of the exfoliated teeth showed that topical fluoride applications caused an increase of approximately 50 p.p.m. in the fluoride concentrations of the outer portion of the enamel. There was no detectable decrease of fluoride from the treated enamel during the period 3-7 years following the fluoride treatment.

REVIEW OF LITERATURE

A COMPARISON between fluoride content of enamel and caries susceptibility has been made in animal experiments, but no such experiments have been reported with regard to human teeth. However, attempts have been made to measure the uptake of fluoride by enamel from fluoride solutions applied topically to human teeth *in situ*. Armstrong found no detectable increase in the concentration of fluoride in the entire enamel of fluoride treated teeth (ARMSTRONG and KNUTSON, 1945). Other determinations of fluoride in the entire enamel of teeth receiving topical treatments have given results which varied too much to be readily interpretable (KIMMELMAN and FOSTER, 1955; HELD, PIGUET and ROESGEN, 1951). Isotope studies have shown that the reaction of fluoride with intact teeth is limited to the surface layer, suggesting that increase in fluoride following topical application may be expected only in the external portions of the enamel (BRUDEVOLD *et al.*, 1957). Chemical analyses of teeth treated with fluoride have confirmed this hypothesis. SYRRIST (1949) determined fluoride in outer portions of enamel of human teeth which had been topically treated, and demonstrated greater uptake from 4% than from 2% NaF solutions. BRUDEVOLD, STEADMAN, GARDNER, ROWLEY and LITTLE (1956) also found that the external portion of the enamel acquired more fluoride from high than low concentrations of topically applied fluoride while there was no significant uptake by sub-surface enamel. The results of these studies are summarized in Table 1.

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TABLE 1. CONCENTRATIONS OF FLUORIDE (p.p.m. OF ASH WEIGHT) IN ENAMEL OF TEETH TREATED WITH NaF AND UNTREATED CONTROLS

Agent	Treatments		Sampling	Fluoride p.p.m. experimental	Control	Investigation
	No.	Period (min)				
NaF 2%	4	5	Outer portion	620*	430	SYRIST (1949)
NaF 4%	4	10	Outer portion	1100*	570	
NaF 1%	1	20	First outer layer	1440**	924	BRUDEVOLD, STEADMAN <i>et al.</i> (1956)
			Second outer layer	561	400	
NaF 0.02%	1	20	First outer layer	968**	777	
			Second outer layer	508	445	

* Fluoride exposure *in situ* once a week.** Fluoride exposure *in vitro*.

The present study was undertaken to determine the increment of fluoride in surface enamel associated with a known reduction in dental caries and to find out whether the concentration of fluoride in the surface enamel of untreated teeth and teeth treated with fluoride solutions would change with time. The outer portion of enamel from deciduous teeth which were shed during and several years after a topical fluoride application study was analysed for fluoride and the results are discussed in an attempt to explain the caries inhibition revealed in the clinical study.

METHOD

The clinical study which extended over 3 years was undertaken at the Eastman Dental Clinic in Stockholm, a city where the water contains only 0.09-0.11 p.p.m. of fluoride. Since a detailed account of the study has been published elsewhere (SUNDVALL-HAGLAND, 1955) only a brief summary of the clinical procedures will be given here. The experimental group included 107 children with a median age of 2 years and 8 months. The teeth on one side (upper and lower) were given four topical treatments with 2% NaF within a period of 15 days, each treatment lasting for 4 min. The untreated teeth and the teeth of a group of 102 comparable children who received no fluoride treatment served as controls. The teeth were thoroughly cleaned with pumice and linen strips before the first application and debris was removed with hydrogen peroxide prior to the three subsequent applications. Dental examinations were given at the beginning of the experiment and 1, 2 and 3 years thereafter.

Teeth which exfoliated or were extracted during and after the study were collected and sent to the Eastman Dental Dispensary, Rochester, New York, for sampling and analysis. All the teeth were deciduous and most of them were anterior teeth. They were grouped according to treatment and time elapsed since the fluoride treatment as shown in Table 2. A great number of teeth was needed in each group to provide sufficiently large samples of enamel for the fluoride analyses. It was possible to divide

the teeth collected during 1953 (4 years after the fluoride treatment) into two groups and thus obtain duplicate results.

Two successive layers of enamel were ground and pooled from teeth of each group, using previously described procedures (BRUDEVOLD, GARDNER and SMITH, 1956). The outer layer was ground as thin as possible (0.1–0.2 mm), while the second layer represented the bulk of the enamel. The samples were separated from dentine contamination by the Manly-Hodge flotation method (MANLY and HODGE, 1939). Samples of coronal dentine from the dentino-enamel junction region were prepared from a few of the tooth groups. Two different procedures for determining fluoride were employed. The samples from the third-year and of one group of the fourth-year teeth were analysed according to the Smith-Gardner modification of the method of Williams (SMITH and GARDNER, 1950). The remaining samples were analysed in duplicate in ARMSTRONG's laboratory using the newly developed Singer-Armstrong micro-method (SINGER and ARMSTRONG, 1956) applicable to determinations on small quantities of material.

TABLE 2. FLUORIDE CONCENTRATION (p.p.m. ASH WEIGHT) OF OUTER AND INNER PORTIONS OF ENAMEL AND DENTINE OF DECIDUOUS TEETH WHICH RECEIVED FOUR TOPICAL APPLICATIONS OF 2% NaF AND OF UNTREATED CONTROL TEETH

	Period of time after fluoride treatment									
	3 years		4 years				5 years		6–7 years	
	F	C	F	C	F	C	F	C	F	C
No. of teeth	16	16	29	23	39	41	22	29	23	18
Enamel Layer 1	151	95	143	81	165	125	155	110	185	130
Enamel Layer 2	37	58	52	49	50	30	35	30	50	40
Dentine	88	107	104	100	—	—	—	—	—	—

F = fluoride treated.

C = control.

RESULTS

The results of the fluoride analyses are given in Table 2. In all the fluoride-treated teeth the fluoride concentration in the outermost portion of the enamel was approximately 50 p.p.m. greater than that found in the controls. There was no such difference in the second layer of enamel nor in the dentine. There was no evidence of decrease of fluoride from the outer or inner portions of enamel with time in either the treated or untreated teeth. In fact, the highest concentrations of fluoride were found in the teeth which had been in the mouth for the longest period of time.

From Table 3 it will be noticed that the topical fluoride treatments caused a slight but consistent reduction in caries. Calculated from the caries figures of treated and

untreated teeth in the same mouth the reductions were 19, 14 and 7.5 per cent after 1, 2 and 3 years, respectively. Comparison of the treated teeth with similar teeth in children receiving no fluoride treatment gave figures of 34, 23 and 12 per cent reduction. The data suggest a slight caries reduction in the untreated teeth of the experimental group, perhaps because of seepage of fluoride from the fluoride treated to the untreated teeth. An insufficient number of teeth was available to test this hypothesis by chemical analysis.

TABLE 3. NUMBER OF NEW CARIOUS SURFACES PER SUBJECT IN TEST AND CONTROL GROUPS* AT 1, 2 AND 3 YEAR EXAMINATIONS. CARIES INHIBITION CALCULATED FROM THE MEANS OF (a) TREATED AND UNTREATED TEETH IN THE SAME MOUTH (b) TREATED TEETH IN TEST GROUP AND CORRESPONDING TEETH IN THE CONTROL GROUP

Period (years)	Test group		Control group	Caries inhibition (a)	Caries inhibition (b)
	Treated side	Untreated side	One side		
1	4.6	5.7	7	19 %	34%
2	7.5	8.7	9.8	14 %	23%
3	9.9	10.7	11.3	7.5%	12%

* The side in the control group corresponding to the control side in the test group.

DISCUSSION

A comparison of Tables 1 and 2 shows that SYRRIST (1949) and BRUDEVOLD, STEADMAN, GARDNER, ROWLEY and LITTLE (1956) obtained considerably greater increase in enamel fluoride from fluoride treatments than was obtained in the present study. This may be due in part to differences in experimental procedures but undoubtedly also to the fact that their studies were concerned with permanent teeth which are known to acquire more fluoride than deciduous teeth.

A direct correlation between the caries score and the fluoride data can be made only with regard to the third year and later data (Table 2), since no teeth were available for chemical analysis during the first 2 years of study. In the third year the caries inhibition was less than half that observed in the first year (12 compared to 34 per cent). The question may be raised as to whether the decrease in caries inhibition with time was related to a gradual decrease or loss of fluoride in the enamel.

Radioactive studies have shown that a portion of topically applied fluoride will wash away quickly while the remainder of the fluoride is retained on the enamel surface (BRUDEVOLD *et al.* 1957). Therefore, except for the immediate loss of excess fluoride, further decrease in enamel fluoride is unlikely. In fact, analyses of young and old teeth have shown that there tends to be a slight post-eruptive increase in fluoride in the external enamel with advancing age, even in teeth from communities (such as Stockholm), with only traces of fluoride in the water supply (BRUDEVOLD, GARDNER and SMITH, 1956). The present finding that the fluoride level was maintained in both treated and untreated teeth during the period 3-7 years after the fluoride

applications is therefore consistent with data in the literature. These considerations and certain concepts concerning the mechanism of fluoride acquisition by calcified structures to be discussed later, suggest that the caries inhibition afforded by the topical applications stems mainly from that portion of the absorbed fluoride which was firmly fixed, and that there was no decrease in this fluoride either during the first two or during subsequent years of the study.

According to this concept the caries reduction observed during the first years following the topical fluoride treatments may result from retarded penetration through the external layer of the treated enamel by the carious process. Once this layer has been pierced by the carious process it is believed that further progress of the lesion will be unaffected by the original fluoride treatment. Hence a decrease in caries inhibition with time is in accordance with this concept.

Because of the high concentration of fluoride in the application solution (2% NaF) calcium fluoride must first have been formed on the enamel surface (NEUMAN *et al.*, 1950). However, there is no evidence that this salt will be permanently retained on the tooth surface since it is slightly soluble and it will provide fluoride ions which readily react with the apatite crystals. Recent studies have shown that these crystals are surrounded by a layer of surface-bound water, the hydration shell (NEUMAN, TORIBARA and MULRYAN, 1953), and that the reaction between the crystal and fluoride takes place in three phases: (1) fluoride ions diffuse rapidly into the hydration shell. This reaction is reversible and fluoride is not firmly fixed. (2) Fluoride then reacts with the surface of the crystal by exchanging positions with surface-bound hydroxyl groups. The rate of this reaction is slower than that of phase 1, and the attached fluoride is firmly fixed. (3) Fluoride may finally penetrate into the crystal and exchange with interiorly located hydroxyl groups. This reaction is exceedingly slow and the low concentrations of fluoride found in bone and teeth suggest that it does not occur to any extent during a lifetime. In fact, analyses of anthropological material have shown that, even after one million years of fluoride exposure, saturation with fluoride, i.e. penetration of the apatite crystals, may be incomplete (OAKLEY, 1946-1948).

From the foregoing it follows that penetration of fluoride into the bulk of the enamel must be mediated through the water of hydration and not by diffusion through the crystals proper. In fully calcified enamel, crystals are packed closely together and are separated only by infinitesimal spaces (SCHMIDT, 1934). Conditions for ionic transfer are, therefore, unfavourable. However, in the partly decalcified enamel of initial carious lesions spaces between the crystals are greater, as has been demonstrated by polarizing microscopy (KEIL, 1937). The increased hydration of carious enamel will facilitate diffusion of fluoride, a fact verified by radioactive studies (MYERS, HAMILTON and BECKS, 1952). But even when there is appreciable penetration of fluoride, the reaction involved remains essentially limited to the surface of the crystals. The fact then that fluoride acts somewhat as a coating agent explains how topical fluoride applications produce only a slight increment in the concentration of fluoride and also how such small increments may provide protection against dental caries.

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THE ORAL ACTINOMYCETES

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Abstract—The mouth appears to be the normal habitat of a large proportion of recognizable genera of Actinomycetes, and in many cases the main or even sole source. The most important are *Actinomyces*, *Leptotrichia*, *Fusobacterium* and *Nocardia*; only the last has saprophytic representatives. It is probable that these oral parasites are descended, by a degenerative evolutionary process involving loss or reduction of Gram-positivity, catalase production and sporulative power, from the free-living Actinomycetes.

INTRODUCTION

It is a remarkable fact, and one that is rather less well recognized by dental bacteriologists than it might be, that the mammalian oral cavity appears to be the natural habitat of a very high proportion of recognizable genera of the "higher bacteria"; that is to say, the branching, Gram-positive, filamentous Actinomycetes. The most important free-living members of the group are the *Streptomyces*; these are superficially mould-like with a vegetative and an aerial mycelium. Under the misnomer of *Actinomyces*, which properly belongs only to the oral parasite (HARTZ, 1877), the *Streptomyces* have sometimes been claimed to be true fungi, appertaining to the Fungi Imperfecti, and they are still so classified by many botanists; however, they are distinctly bacterial, not only in size, but in their nuclear cytology (KLIENEGER-NOBEL, 1947) and in the chemical composition of their cell walls (CUMMINS and HARRIS, 1958). At the same time, they differ fundamentally from the true moulds in that their entire life-cycle is completed in one generation, whereas the Fungi Imperfecti are actually the vegetative stages of mould-like fungi of which the sexual stages are either suppressed or so far unrecognized. There is thus little room for doubt that the streptomycetes are truly bacterial in their affinities, and also, for reasons that will be explained, that they are the free-living representatives of the oral genera which are the subject of this paper. Of these there are three, *Actinomyces*, *Leptotrichia* and *Fusobacterium*, which consist mainly, if not exclusively, of specialized parasites of the mouth and associated tissues. Among the oral Actinomycetes, only *Nocardia* can claim a wide distribution in nature apart from this habitat.

CLASSIFICATION OF THE ACTINOMYCETES

The monograph of WAKSMAN (1940) refers to fourteen different classifications of the Actinomycetes, and this by no means exhausts the possible list. The most influential and valuable of these is that of ØRSKOV (1923), by whom the confusion in the application of the name *Actinomyces* was clearly resolved, but it is unfortunately not

possible to claim that this confusion was finally cleared away at that time, or indeed that it has ever been finally cleared away. In WAKSMAN's own classification (1940) the terminology at least was less clear than might have been wished, whereas so recently as 1957 a classification was proposed by TEŠIĆ (1957) in which no distinction at all was drawn between *Streptomyces* and *Actinomyces*.

There is still no generally accepted system which embodies the most recent information available upon the morphology of filamentous bacteria, and the definitions which we suggest here are considerably influenced by, and in some cases largely founded upon, our own observations and experience, as well as those of other workers in this laboratory.

The Order *Actinomycetales* contains the saprophytic genera *Streptomyces*, *Micromonospora* and *Waksmania*, of which the first-named produces its spores in long chains, the second, singly, on short branches, and the last is intermediate in this respect, producing very short chains, usually of two spores, also on side-branches. The true *Actinomyces* resembles *Micromonospora* in its mode of sporulation (MORRIS, 1951a), but shows obvious signs of degeneration, as befits its parasitic habit (BISSET, 1957); the two, rather dissimilar micro-organisms which are classified as *Leptotrichia* appear to have lost almost all their powers of sporulation, but such traces as they possess are again suggestive of a degenerative descent from *Streptomyces* (BAIRD-PARKER and DAVIS, 1958); *Fusobacterium* probably represents a further stage of descent; *Nocardia* species possess a well-marked life-cycle, quite different from any of these; but the name of this genus has also been misapplied.

The physiology of these bacteria does not lend itself readily to the biochemical tests which are of such value in the classification of many other groups, but it is of importance that whereas the saprophytic genera are invariably aerobic the parasites have a distinct tendency towards microaerophilia and even anaerobiosis. This tendency is commonly found among members of Gram-positive bacterial families which have adopted a parasitic habit of life, and may well be a genetic accident to which they are prone. Truly Gram-negative anaerobes are a rarity, and the examples most commonly quoted have obvious Gram-positive affiliations. Loss of Gram-positivity is also a concomitant of parasitism.

THE ORAL PARASITES

(A) *Actinomyces*

The name *Actinomyces* was first applied by HARTZ (1877) to the "ray fungi" causing the disease of cattle and man which, in consequence, came to be called actinomycosis. The name was thus descriptive of the typical lesions, and its application to the saprophytic *Streptomyces* owes something to the erroneous belief that the appearance of these lesions around sharp pieces of grain embedded in the tissues of the jaw indicated that the infection was derived from the grain, from which such "aerobic actinomyces" could readily be isolated. However, it was early realized that this was a mistake, although, as already stated, the confusion of nomenclature has been slow to die. The true *Actinomyces* was isolated from clinically healthy human mouths by BERGEY (1907) and by LORD (1910); the latter claimed to have proved

the pathogenicity of his isolates. Since that time further, similar isolations have frequently been made, but the morphology of *Actinomyces* was not properly described in detail before MORRIS (1951a), whose account of the life-cycle of *A. bovis* was confirmed by BATTY (1958) in *A. odontolyticus*, a species which she found to be almost universally present in human mouths.

As already suggested, the life-cycle most closely resembles that of *Micromonospora* among the saprophytes; the spores are single and borne individually on short side-branches arising from a fairly complex secondary mycelium. The primary mycelium, to which the germinating spores give rise, however, is by contrast very simple; branching occurs, but it is not at all profuse and is often impermanent, proceeding to cell division and fission at the point of branching. This may reasonably be considered to be a degenerative character in these particular circumstances, and so also may the form of the spore, which is often less globular than the mature spore of *Micromonospora* and more like the immature, lanceolate form (BISSET, 1957). This type of life-cycle may take several weeks to complete, and in consequence most observers are familiar only with the vegetative stages which are quite undistinguished, resembling corynebacteria or nocardias rather than the more elaborate forms.

Aerobic actinomyces are often described in the mouth; for these there are two distinct explanations. The first is that occasional strains of true actinomyces are so little sensitive to oxygen that they can grow quite readily under conditions where no particular care has been taken to reduce the tension; the second, and more frequent one is that the bacteria so described are correctly assignable to other genera, especially *Nocardia*.

(B) *Leptotrichia*

The commonest organism visible by microscopic methods in dental material is *Leptotrichia buccalis*. Its characteristic filaments, in pairs, with the distal ends slightly pointed, were among the first recognizable bacteria to be figured by Antonj van Leeuwenhoek. The name was devised by TREVISAN (1879), and although there has been considerable confusion in its application in this case also, there is no doubt of the identity of the very characteristic micro-organism on which it was conferred. Previously, such names as *Leptothrix*, *Streptothrix*, *Cladothrix*, etc. had been used to denote almost any filamentous micro-organism, although all or most of them are correctly applied to autotrophic iron bacteria. After the general acceptance of *Leptotrichia* for the oral genus it was applied rather too freely, to a variety of more-or-less filamentous bacteria associated with inflammatory conditions of the conjunctiva, most of which have little apparent relationship with *L. buccalis*, as well as to what we now consider as the second species of the genus, *L. dentium* (BAIRD-PARKER and DAVIS, 1958). This organism was first described by FENNEL (1918), as *Streptothrix interproximalis*, and it has also been a source of confusion in nomenclature because it has been confused with *L. buccalis*.

In fact these two species are rather less alike than might be wished, even for inclusion in a single genus, so that an acceptable definition of *Leptotrichia* cannot easily be framed to cover them both; however, they have many points of resemblance,

and little service would be paid to the clarification of the situation by insisting upon a new generic name within the family *Actinomycetaceae*. As we have already suggested, in the Introduction, they may be considered to represent progressive stages in the degenerative evolution of parasitic forms from a streptomyces-like ancestor (see Table 1). Of the two, *L. dentium* is the more primitive, in that it is Gram-positive, branches freely and occasionally produces chains of spores, although there is no sign of an aerial mycelium (BAIRD-PARKER and DAVIS, 1958); it also produces catalase and can grow freely under aerobic or anaerobic conditions. The most characteristic morphological feature of *L. dentium* is the "whip-handle". This is the name given to the appearance produced when the stout ($2.5\ \mu$) bacillary forms germinate at the poles and points of division to produce long filaments of about half their own diameter, and it is quite accurately descriptive. This, like the occasional spore production, may be a relict of the condition in streptomyces, where the smaller vegetative and larger aerial mycelia alternate in the life-cycle.

In *L. buccalis* Gram-positivity is lost, except in young cultures, the longer filaments are less frequent and rarely form branches, never spores; their maximum diameter is less. Catalase is not produced, so that it is sensitive to oxygen at atmospheric concentrations; some isolates are entirely anaerobic initially. It grows mainly in the form already described, as long, paired, fusiform bacilli.

Because of this morphology and its tendency to anaerobiosis *L. buccalis* has sometimes been confused with *Fusobacterium*. The latter genus represents (on the same hypothesis) an even further stage in adaptation to parasitism, and Table 1 shows the relationship between it and the two species of *Leptotrichia* in respect of the characters of morphology and physiology which have been under discussion.

TABLE 1.

	<i>Leptotrichia dentium</i>	<i>L. buccalis</i>	<i>Fusobacterium</i>
Gram-staining	Always positive	Positive in young cultures	Always negative
Width	1-2.5 μ	1-1.5 μ	0.3-0.6 μ
Typical form	Filamentous with "whip-handles"	Fusiform or filamentous	Fusiform or filamentous
Branching	Frequent	Infrequent	Never
Oxygen sensitivity	Aerobic	Micro-aerophilic	Anaerobic

(C) *Fusobacterium*

The name was applied by KNORR (1922) to the organism described by VINCENT (1896) and long known as *Bacillus fusiformis*. Although it has been variously classified in the *Bacteriaceae* and *Parvobacteriaceae* by different systematists, the

present theory that it belongs truly to the Actinomycetes is not new, but has already been advanced by KLUYVER and VAN NIEL (1936), PRÉVOT (1938) and BISSET (1951).

Fusobacterium has not only been confused with *Leptotrichia buccalis*, but also (in the manner which readers of this paper will by now recognize as inevitable in this field of study) with such diverse organisms as spirochaetes and the protozoon *Selenomonas*. It is very probable that most of the reports attributing motility to members of the genus are due to such misidentifications. One authority (PRÉVOT, 1940) separates the motile forms under the generic name *Fusocillus*, but if such a genus is in fact justifiable it lies outside the scope of this section.

Two species are distinguishable, corresponding to the *Fusobacterium nucleatum* and *F. polymorphum* of KNORR (1923); several other named species are apparently synonyms for these and for *Leptotrichia buccalis*. The two species are morphologically similar; they are fusiform with occasional unbranched filaments, Gram-negative and characteristically show a granular appearance, sometimes suggestive of nuclear structures, but not definitely known to be of this nature. They are strictly anaerobic.

Fusobacteria have often been described in a pathogenic role, but usually as concomitants of other pathogens; they do not appear to be either virulent or invasive.

(D) *Nocardia*

Alone among the oral Actinomycetes the *Nocardia* are not specialized parasites but members of one of the most versatile of all bacterial groups. They occur in pathogenic conditions (when they are usually referred to by pathologists as aerobic actinomycetes), in soil, as symbionts of insects and in many human mouths. The name was first applied by TREVISAN (1889) to one of the veterinary pathogens, and the general morphological characters of the genus have long been known, but several recorded species have aerial mycelia and spores of a distinctly streptomycetic type, and should not, in our opinion, be included in the genus.

The fullest description of the morphology of *Nocardia* is that of MORRIS (1951b). The Gram-positive filaments are septate and branch only temporarily, the branch soon becoming detached. All or any of the cells in the filament may become transformed into coccal bodies, which later become resistant spores. The cytological details are more like those of the eubacteria than of the more complex Actinomycetes, and it is not unlikely that *Nocardia* is a primitive, rather than a degenerate, form.

(E) *The Leptothrix theory*

Among the filamentous bacteria described from the human mouth are two rather remarkable organisms, which it would not be correct to omit completely from this account; these are "*Leptothrix racemosa*" (VICENTINI, 1897) and "*L. falciformis*" (BEUST, 1937). The names are, of course, invalid.

Both were observed in tooth-scrapings, and neither has been isolated and grown successfully *in vitro*. They are described as relatively thick filaments, the ends of which have attached to them masses of coccal cells ("*L. racemosa*") or fusiform elements ("*L. falciformis*"), thus simulating fruiting heads of fungi. The latter form has also been described as "*Vibriothrix tonsillaris*" or, less portentously, as "the test-

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tube brush organism". Such appearances are quite often seen in microscopic preparations of the materia alba, and have been claimed to be the true sources of the smaller, typical bacteria of the mouth. These, the originators of this theory consider to be simply the spores or mycelial fragments of the "leptothrices". Whether or not these organisms have any real existence as such is problematical, but it can be stated quite unequivocally that this theory of the origin of the bacterial oral flora is not generally acceptable to those who have studied it. The explanation proposed by THJØTTA, HARTMAN and BØE (1939) and also by DAVIS and BAIRD-PARKER (1959) for these supposed fruiting heads is that they represent filaments of *Leptotrichia* to which smaller bacteria have become attached, and which are profiting mutually by the nutritional association.

IMPORTANCE OF THE ORAL ACTINOMYCETES

None of the infections of the teeth and associated tissues appears to be simple in its aetiology. Not only do bacteria combine in complex physiological interrelationships, but their pathogenic activity is greatly influenced by imponderable factors in the environment and constitution of the host. In almost every case, the most that can be stated is that certain bacteria are associated with a condition. So far as periodontal disease is concerned, many of the bacteria described above have been incriminated as agents, either direct (*Fusobacterium*) or through their part in the formation of subgingival calculus (*Actinomyces*, *Leptotrichia*, *Nocardia*). The problem of the role of bacteria in dental caries is an exceedingly vexed one, and would require considerably more than the space here at our disposal for even a brief review. However, some part of the responsibility for this also has been claimed for Actinomycetes, at least in advanced conditions, and it has also been suggested that they are concerned in the production of the brown pigmentation of dentine.

The only member of the group which can be claimed unequivocally to be pathogenic is *Actinomyces bovis* (or *israelii*), the cause of actinomycosis, and this is fortunately a rare disease in man.

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CURRENT PAPERS IN ORAL BIOLOGY

Honorary Editor: MAURICE V. STACK

In recent years it has become increasingly difficult for the dental research worker to examine all the published work that may be of interest to him. This is partly because research papers in the broad field of oral biology are published in many journals other than those devoted specially to this subject, and partly because both the methods and the results of research in many disciplines may have an important application to dental and oral problems.

The *Archives of Oral Biology* will, therefore, provide a special section in which will be listed the titles and references to relevant research papers in a wide variety of Journals published throughout the world. It is intended that this section will provide a means whereby the specialist will know promptly of the existence of relevant papers published throughout the world. The selection will be made by experienced research workers who will include not only papers directly related to oral biology but also work which may have an indirect bearing on this field. For the convenience of readers, the papers will be listed under a limited number of broad headings, and below are given the headings that will be used together with brief notes indicating the types of research to be included under each heading:—

- (1) **Anatomy.**
- (2) **Histology, Normal,** including electron microscopy, histochemistry, microradiography and autoradiographic studies on normal human and animal tissues, and studies on the growth of such tissues in tissue culture.
- (3) **Physiology.**
- (4) **Biochemistry.** The term is interpreted liberally and includes papers on such studies as the solubility of calcified tissues, the determination of crystal structure in tissues by X-ray diffraction, etc.
- (5) **Genetics.**
- (6) **Histopathology** including electron microscopy, histochemistry, microradiography, and autoradiographic studies on *naturally occurring* lesions in human and animal tissues. The behaviour of such diseased tissue in tissue culture. (Studies on *experimentally induced* lesions, except caries, are listed under EXPERIMENTAL PATHOLOGY).
- (7) **Experimental Pathology.**
- (8) **Caries.** Included under this heading are all papers on caries from whatever aspect the disease is considered, although only papers *directly* bearing on caries should be included. Fundamental studies which have an *indirect* bearing on caries should not be included under this heading, e.g. a paper on "The uptake of fluorine by powdered enamel" should be listed under BIOCHEMISTRY but a paper on "The effect of fluorine on the rate of development of carious lesions" should be listed under CARIES.
- (9) **Microbiology** including investigations with antibiotics and other microbial substances.
- (10) **Epidemiology and Clinical Studies.** Only clinical studies which contribute to fundamental knowledge are included, i.e. studies on large series, field studies, etc., which often provide valuable information on the distribution, incidence or some other aspect of a disorder. Descriptions of single cases or small groups of cases rarely qualify for listing.
- (11) **Materials and Technique.**
- (12) **Miscellaneous.**

Abbreviated titles of journals published throughout the world from which current papers are to be selected by members of the Honorary Editorial Advisory Board and classified under the twelve headings.

Acta med. hung.
Acta med. scand.
Acta microbiol. Acad. Sci. hung.
Acta odont. scand.
Acta path. microbiol. scand.
Acta physiol. Acad. Sci. hung.
Acta Sch. med. Univ. Kioto
Advanc. Carbohydr. Chem.
Advanc. Enzymol.
A.M.A. Arch. Dis. Child.
Amer. J. Anat.
Amer. J. clin. Path.
Amer. J. Hyg.
Amer. J. med. Sci.
Amer. J. Ophthalm.
Amer. J. Orthodont.
Amer. J. Path.
Amer. J. Physiol.
Amer. J. phys. Anthropol.
Amer. J. publ. Hlth.
Amer. J. trop. Med. Hyg.
Anat. Rec.
Ann. Inst. Pasteur
Ann. Méd.
Ann. trop. Med. Parasit.
Antibiot. and Chemotherap.
Arch. Anat. Physiol., Lpz.
Arch. Path. (Lab. Med.)
Arch. mikr. Anat.
Aust. dent. J.
Bact. Rev.
Biochem. J.
Brit. J. exp. Biol.
Brit. J. exp. Path.
Brit. J. Nutr.
Brit. med. Bull.
Brit. med. J.
Bull. schweiz. Akad. med. Wiss.
Canad. J. Biochem.
Canad. J. Microbiol.
Clin. chim. Acta.
Dtsch. med. Wschr.
Dtsch. zahnärztl. Z.
Dtsch. Zahn-, Mund- u. Kieferheilk.
Exp. Cell Res.
Fed. Proc.
Fogorv. Szle.
Growth
Guy's Hosp. Rep.
Helv. physiol. pharmacol. Acta
Helv. odont. Acta
Hum. Biol.
Int. dent. J.
J. Amer. dent. Ass.
J. Amer. med. Ass.
J. Anat., Lond.
J. appl. Physiol.
J. Bact.
J. biol. Chem.

J. biophys. biochem. Cytol.
J. clin. Med.
J. dent. Ass. S. Afr.
J. dent. Res.
J. exp. Med.
J. gen. Microbiol.
J. Hyg., Camb.
J. Immunol.
J. infect. Dis.
J. Lab. clin. Med.
J. Pediat.
J. prosth. Dent.
J. Path. Bact.
J. Physiol.
J. S. Calif. dent. Ass.
Lancet
Leeuwenhoek ned. Tijdschr.
Med. J. Aust.
Med. J. S. Afr.
Nature, Lond.
N.Y. St. dent. J.
N.Z. dent. J.
N.Z. med. J.
Odont. Revy
Odont. T.
Odont. Chilena.
Orv. Hétl.
Öst. Dent. Z.
Öst. Z. Stomatol.
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Parodontologie
Physiol. Rev.
Pr. méd.
Proc. nat. Acad. Sci., Wash.
Proc. Nutr. Soc.
Proc. roy. Soc.
Proc. roy. Soc. Med.
Proc. Soc. exp. Biol., N.Y.
Proc. zool. Soc. Lond.
Quart. J. exp. Physiol.
Quart. J. Med.
Quart. J. micr. Sci.
Quart. Rev. Biol.
Rev. Asoc. odont. argent.
Rev. Asoc. paul. Cir. Dent.
Rev. dent. Santiago.
Rev. mens. suisse Odont.
Schweiz. med. Wschr.
Schweiz. Mschr. Zahnheilk.
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Yale J. Biol. Med.
Z. mikr-anat. Forsch.
Z. Zellforsch.

A HISTOLOGICAL STAINING METHOD FOR SITES OF CALCIFICATION IN TEETH AND BONE

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Abstract—Formalin-fixed tissues are treated with hot absolute alcohol, benzol or pyridine, decalcified if necessary and stained with Sudan black B. The dentine-predentine junction, enamel where it becomes acid soluble, and the edge of the preosseous matrix of bone are stained dark blue, the rest of the calcified tissues being unstained. These areas have, after pyridine treatment, a methylene-blue extinction at a pH less than 2.6 and are under certain circumstances metachromatic. Calcification, as demonstrated by Sudan black staining, is slowed in rickets and immediately resumed after vitamin D therapy. It is concluded that a specific type of acid mucopolysaccharide is involved.

It is well known that Sudan black B will stain the granules of mast cells (MONTAGNA and NOBACK, 1947; WISLOCKI and SOGNAES, 1950) but, under ordinary methods of fixation, the mucopolysaccharides of teeth, cartilage and bone are unaffected by this stain. In an endeavour to modify mucopolysaccharides so that they would take up Sudan black, use was made of the finding reported by HADADIAN and PIRRIE (1948) that pyridine precipitates hyaluronic acid. When these tissues were treated with pyridine, unexpected results were obtained which are described and discussed in this paper. Preliminary accounts of some of these findings have already been published (IRVING, 1958a, 1958b).

MATERIAL AND METHODS

Material

This was from normal, foetal, young and adult rats and from normal, approximately 3 month old human foetuses. In addition some experiments were conducted with rachitic rats. The rats were killed with coal gas or intraperitoneal Nembutal, the heads were cut off and the lower jaws removed.

Histological methods

Rats. The skulls were bisected and fixed in formol-saline, BAKER'S formol-calcium (1946), absolute alcohol or Bouin's solution. In some cases the upper incisors were dissected out with the surrounding alveolar bone. Of these some were mounted in gelatin and the formative end, which was sufficiently soft to be cut without decalcification, was cut in transverse section on the freezing microtome. Others were treated with pyridine in accordance with BAKER'S method for extracting lipids (1946): 2 hr at room temperature, with a change at 1 hr, and then 24 hr at 60°C. After washing they were mounted in gelatin and cut as described above.

Most of the incisor teeth were cut after decalcification in longitudinal section with the surrounding tissues, the molars being included in the section if possible. Some were decalcified with ethylenediamine tetra-acetic acid (EDTA) or nitric acid-formalin mixture, and then embedded in paraffin or gelatin. Most of the teeth were treated with pyridine, as described above, before decalcification, embedded in gelatin and cut on the freezing microtome. Some were treated with hot alcohol or hot benzol (after dehydration with alcohol) instead of with pyridine.

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Human foetal material. The mandibles were removed, fixed in formalin, treated with pyridine as above, decalcified with nitric acid-formalin mixture, cut into small segments and embedded in gelatin. The central lower incisor was cut in the labio-lingual plane.

In all cases when nitric acid decalcification was used, the tissues were soaked for 1 day afterwards in 5% sodium sulphate solution. The tissues were well washed in running water after each stage of the procedures used.

Staining methods

Sudan black B (George T. Gurr). BAKER's method was used (1949). The sections were put into 70% alcohol and were then stained for 2-3 min with a saturated solution of Sudan black in 70% alcohol. Following a brief rinse in 70% alcohol, they were treated for 1 min with 50% alcohol. After washing in several changes of distilled water, they were mounted in glycerine jelly. If, after staining, the sections were dehydrated and mounted in balsam, the staining with Sudan black was dissolved out unless dehydration was very quick; if the sections were taken back to 70% alcohol, they could be restained as before with Sudan black.

Toluidine blue. Sections were stained with 0.25% toluidine blue in 0.25% borax solution for 15 sec, and were examined mounted in water. Such preparations were not permanent. Some tissues were fixed in 4% basic lead acetate which has been recommended when staining for metachromasia.

Other methods. The methylene-blue extinction method as described by PEARSE (1953) was employed using a sodium acetate-sodium barbiturate buffer at pH values from 2.6 to 7.2. The perchloric-acid technique for the removal of ribonucleic and desoxyribonucleic acids, Hale's colloidal iron and the alcian blue methods, were also used, all following PEARSE's instructions. The periodic acid-Schiff reaction was used in some cases and most sections were routinely stained with haematoxylin and eosin. Some of the sections were incubated in pepsin solutions at pH 1.2 for 24 hr. BENDITT and FRENCH (1953) found that N/10 sodium hydroxide removed hexosamine from tissues. Some sections were therefore incubated in N/10 sodium hydroxide for 2½ hr at 37°C.

Hyaluronidase. The enzyme solution used was made by dissolving "Hyalase" (Benger Laboratories, Ltd.) in 0.1 M acetate buffer at pH 5.5, at a concentration of 1500 I.U. of testicular hyaluronidase per millilitre. Sections were incubated in this solution for 2½ hr (following the technique of BENDITT and FRENCH, 1953), controls being incubated in buffer solution only.

Rickets. Some experiments were performed with rachitic rats. The diet used was a modification of the STEENBOCK and BLACK diet No. 2965 (1925), which had been previously used by the writer (IRVING, 1944), with a high P and a low Ca content. This diet causes more drastic tooth changes than the conventional high Ca low P diet (GAUNT and IRVING, 1939).

RESULTS

Sudan Black Staining

Rat tissues

When these were stained without preliminary treatment with pyridine, no specific staining of either the teeth or surrounding alveolar bone was seen, all being a pale blue. After treatment with pyridine, the following results were found.

Dentine. Calcified or decalcified sections of dentine: a narrow zone, staining intensely with Sudan black, at the dentine-predentine junction, on both the labial and lingual sides, with staining extending sometimes a little distance up the dentinal tubules (Fig. 1). The calcospherites which are often found at the dentine-predentine junction also stained intensely. The predentine did not stain at all, nor did the dentine except for the part mentioned above.

Enamel matrix. This was studied only in decalcified sections. The newest formed enamel was colourless, but at the area where the enamel began to become acid soluble it stained strongly with Sudan black (Fig. 1). This appearance was better seen with EDTA decalcification, since after nitric acid the newer enamel tended to have a pale-green colour.

Alveolar bone. In all places where active apposition was occurring, a narrow, dark-blue line was found, not on the edge of the bone, but a very short distance

inside the bone (Fig. 2). The distance of the line from the edge of the bone varied somewhat from specimen to specimen, presumably depending on the speed of apposition. For convenience this line has been called a "calcification line".

Human foetal material

The dentine and alveolar bone stained in exactly the same way as the rat tissues. The teeth were too young for enamel calcification to have started.

For Sudan black to stain in this way, it was essential that treatment with pyridine preceded decalcification, though decalcification was not necessary. It was not heat alone which caused the staining reaction, since heating the tissues in formalin for 24 hr at 60°C had no effect, nor was pyridine the only reagent to cause Sudan black staining. After fixation in absolute alcohol, provided it was of sufficient duration, or treatment with hot alcohol, or hot benzol after alcohol or formalin fixation, the dentine-predentine junction and calcification lines in bone stained as after pyridine, but the enamel matrix now stained in its full length. After alcohol treatment only, a great deal of other sudanophil material was present in the pulp and surrounding tissues, a fact which rather complicated the picture. It was thought possible that simple dehydration was the cause of the Sudan black staining, and an undecalcified section, not pyridine extracted, was dried out at 60°C for one day and then stained. However, no Sudan black staining was seen. Embedding in paraffin after treatment with pyridine and after decalcification prevented Sudan black staining completely. N/10 sodium hydroxide did not abolish the Sudan black staining.

In routine practice pyridine was always used, as it was quicker and more convenient than alcohol or benzol, the only objection being its unpleasant odour.

Metachromasia

Since it appeared possible that the sudanophil material was of mucopolysaccharide nature, toluidine blue staining was carried out on tissues treated by all the various techniques described above. As is well known, great caution must be employed in interpreting results on decalcified material, since metachromasia may be completely changed by decalcification (HELLER-STEINBERG, 1951). In addition, there is still dispute as to whether sections stained with toluidine blue should be examined in water or after hydration, when the staining is considerably altered. BÉLANGER (personal communication) has found that the results, when following the synthesis sulphomucopolysaccharides using S³⁵, can only be duplicated with toluidine staining if the sections are examined in water or crown oil. PEARSE (1953) has also condemned the use of alcohol. The present writer therefore examined all his sections in water.

The chief aim was to see if there was any correspondence between the staining reactions with Sudan black and toluidine blue. When undecalcified teeth, fixed in formalin, were stained with toluidine blue, it was found that the dentine-predentine junction was intensely metachromatic, predentine was pink and dentine unstained. This has also been reported by LEBLOND, BÉLANGER and GREULICH (1955). Thus in undecalcified teeth, toluidine blue stained the same areas as Sudan black, but in

addition toluidine blue stained the predentine which was not affected by Sudan black. After decalcification, both dentine and predentine were strongly metachromatic, thus obscuring any specific staining at the dentine-predentine junction. In both calcified and decalcified sections, the enamel matrix stained blue up to the point where it began to be acid soluble, when it appeared metachromatic in the same area which stained with Sudan black.

Bone could be investigated only in decalcified sections. With all methods of preparation and fixation, and whether pyridine was used or not, the calcification lines seen with Sudan black staining were strongly metachromatic. However, Sudan black stained the calcification lines only, whereas with toluidine blue the pre-osseous matrix and the rest of the bone were also metachromatic but less so than the calcification lines. The picture obtained with toluidine blue was very similar to that reported by VINCENT (1955) in the osteones of mature bone.

Hyaluronidase

This enzyme had no effect at all in diminishing or abolishing the stainability of the sudanophil material of teeth or alveolar bone in either calcified or decalcified sections, using all the methods of fixation and treatment described above. Nor did it affect the metachromasia of calcified tissues. In undecalcified teeth, the staining of predentine was changed from metachromasia to orthochromasia or the staining was abolished, depending on the fixative used, but the intense metachromasia of the dentine-predentine junction was unaltered.

SCOTT (1950) has reported that formaldehyde is an inhibitor of hyaluronidase. It was found in the present experiments that, provided the section was well washed, activity of the enzyme was not impaired.

Methylene-blue Extinction Method

When this was carried out on formalin-fixed, decalcified material, the extinction for dentine, predentine and enamel was pH 3.7. At pH 4.2 the predentine was stained but the dentine was not, and the enamel was pale green where it became acid soluble. The zone at the dentine-predentine junction did not stain at a pH below 4.6, but stained deeply as a fine line at pH 4.6 and higher values. The calcification lines in bone did not stain at any pH, but the pre-osseous matrix stained at pH 4.6 and higher values.

When tissue was examined after fixation in formalin and treatment with pyridine, hot alcohol or hot benzol, quite a different appearance was seen. In all cases the dentine-predentine junction stained at pH 2.6, the lowest value employed, and at this level neither the dentine nor predentine was stained. The acid-soluble part of the enamel matrix likewise stained in the pH range 2.6-3.7. The calcification lines in bone stained in the pH range of 2.6-3.2, the rest of the bone being unstained.

It will thus be seen that treatment with pyridine, hot alcohol or benzol had the effect of making the dentine-predentine junction and calcification lines of bone not only sudanophilic but also stainable with methylene blue at low pH values.

Miscellaneous Techniques

Periodic acid-Schiff reaction. The dentine was deep red, the predentine pink and no specific staining was seen at the dentine-predentine junction.

Pepsin. Exposure of sections to this enzyme, sufficient to digest all the embedding gelatin, much of the pulp and periodontal membrane and many of the ameloblasts, did not abolish the specific staining with Sudan black.

Perchloric acid. This treatment, to remove ribonucleic and desoxyribonucleic acids, did not change in any way the staining reactions in bone and teeth obtained with Sudan black or toluidine blue.

Hale's colloidal iron and the alcian blue methods. These were carried out on undecalcified teeth and alveolar bone but gave no useful information. With Hale's method all the calcified tissues stained blue and the predentine was unstained. Alcian blue stained predentine and the pre-osseous matrix of the alveolar bone very intensely, but did not stain enamel or dentine. VINCENT (1955) obtained very similar results using these stains on bone.

Changes During Rickets

Since it seemed likely that Sudan black was staining a constituent of the tissue concerned with the initiation of calcification, it appeared of interest to see what would occur in active and healing rickets. Young rats were put on to the modified Steenbock and Black rachitogenic diet for 28 days. Some were then killed and their teeth and oral tissues examined. Others were given one dose of 30 I.U. of vitamin D by mouth and killed at daily intervals up to 8 days later.

Haematoxylin and eosin sections showed that the predentine width was greatly increased in the rats with rickets. Only the dentine-predentine junction was stained with Sudan black, which agreed with the concept that dentine calcification still continued in rickets but at a slower rate than dentine apposition (Fig. 3). The Sudan black staining of the enamel matrix was the same as in normal animals. When vitamin D was given, a haematoxylin-staining line appeared 2 days later in the predentine at a distance of about 16 μ from the pulp, and a sudanophil line appeared in the same position at this time. Concurrently, an intense sudanophilia appeared in the rachitic predentine (Fig. 4) which also began calcifying. The persistence of some sudanophilic material at the end of 8 days (Fig. 5) suggested that this process was not apparently completely finished at that time.

In rachitic alveolar bone, a picture very similar to that in dentine was found. Wide osteoid seams were present, but where they abutted on calcified bone, a sudanophil line was to be seen (Fig. 6), indicating that calcification was still in progress but was much slower than bone apposition. Two days after vitamin D was given, the sudanophilia began to spread from the calcification line into the osteoid, as the osteoid became calcified (Fig. 7). This process, like that in the dentine, must have been a slow one, since a similar appearance was still found 8 days after vitamin D had been given.

DISCUSSION

It is apparent that the original reason for using pyridine in this technique was not correct, since the effect is not specific to pyridine but can also be obtained with other lipid extractants. It was not produced by merely heating the tissues. The chemical change which enables the sudanophil material to become sudanophilic and also to change its methylene blue extinction to a very low pH is not clear. It appears that its precursor must be closely attached to the calcified phase, since it is removed with it unless pyridine treatment comes first. However, even in undecalcified tissues, pyridine is essential for Sudan black staining. No other stain employed, except methylene blue at pH 2-6, gave exactly the same results as Sudan black. While some stained the sudanophil material, they also stained other parts of the section. Sudan IV was tried as another lipid stain but did not duplicate Sudan black staining, nor did the p.a.-Schiff reaction. Why Sudan black stains in this way is not known; the composition and staining possibilities of this dye are still incompletely understood. Absolute alcohol readily removes the stain, but the tissue can be restained; the stain cannot be applied to paraffin sections in spite of preliminary pyridine treatment.

It would appear probable from the similarity of appearance of Sudan black, toluidine blue and the methylene-blue extinction methods, that an acid mucopolysaccharide is being stained, especially when the very low methylene-blue extinction value is considered. Perchloric acid treatment seems to rule out ribonucleic and desoxyribonucleic acids which are regarded as the only other compounds which might react similarly. It is true that GOMORI (1952) views the methylene-blue extinction method with suspicion and, taken alone, it requires caution in its interpretation. When, however, the three methods all stain exactly the same area, one can regard the results with more confidence. Presumably treatment with pyridine or the other compounds unmasks the acid mucopolysaccharide so that it becomes stainable.

The fact that hyaluronidase does not change either the Sudan black or toluidine blue staining of the calcified tissues does not invalidate the supposition that acid mucopolysaccharides might be involved, since, as PEARSE (1953) has pointed out, the enzyme does not act, in many instances, upon mucopolysaccharides in tissue sections. In the present experiments, only the metachromasia of the predentine is altered. HUGHES (1956) has reported that the calcifiability of dentine caps *in vitro* is unaffected by preliminary treatment with hyaluronidase.

It can be said with some confidence that the sudanophil material, after pyridine or other treatment, is only at the site where calcification is being initiated, i.e. at the dentine-predentine junction, in enamel where it is becoming acid soluble, and at the edge of the pre-osseous matrix in bone.

In dentine, Sudan black and toluidine blue stained in exactly the same areas in undecalcified sections. BÉLANGER (1954) has found that S^{35} is laid down at the dentine-predentine junction 2 hr after injection, and LEBLOND *et al.* (1955) have concluded that the bulk of the chondroitin sulphate of dentine is synthesized or at least sulphated at the very zone where predentine transforms into dentine. The only

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peculiar aspect is that both Sudan black and toluidine blue in undecalcified sections stain only this area, although the zone of S^{35} , as seen by autoradiographs, moved progressively "outwards" with accretion of more dentine (BÉLANGER, 1954). Thus there is some difference between the mucopolysaccharide at the dentine-predentine junction and that in the depths of the dentine.

Sudan black and toluidine blue staining agreed well in enamel as far as the site of the respectively staining material was concerned. This was found at the level where acid solubility of the enamel began and the same has been reported as regards metachromasia by WISLOCKI and SOGNAES (1950). The staining also extended to shreds of organic enamel sometimes retained at the incisal end of the tooth. However, BÉLANGER (1955) showed that S^{35} was incorporated into enamel when newly formed and then gradually disappeared, and did not seem to correspond at all with the area that stained with toluidine blue or Sudan black. BÉLANGER has not commented on this lack of correspondence in situation of S^{35} and metachromasia in enamel, which cannot at present be explained.

The pre-osseous matrix in bone has been described by VINCENT (1955) and by SCOTT and PEASE (1957). VINCENT says it is orthochromatic and bounded by a zone of calcified bone (as shown by micro-radiography) which is metachromatic; LACROIX (1956) concludes that "a new layer of bone changes its orthochromasia into metachromasia when it begins to manifest a strong affinity for calcium." As with dentine, BÉLANGER (1954) also found with bone that the zone of S^{35} , which appeared under the periosteum after administration of this isotope, moved away from the surface of the bone with fresh apposition. However, only the edge of the pre-osseous matrix stained with Sudan black. Toluidine blue stains all calcified bone in decalcified sections to a variable degree, but Sudan black is a good deal more specific since it stains only the actual site of calcification. It is of interest that VINCENT found the metachromasia of bone unaffected by hyaluronidase.

The fact that the sudanophil material is concerned with calcification is convincingly shown by the changes seen in rickets. Here, in both dentine and bone, calcification is delayed but not stopped, and both the predentine and pre-osseous matrixes widen, the latter becoming an osteoid seam. When vitamin D is given, calcification of both these zones occurs shortly after, almost explosively in dentine and more slowly in osteoid. As calcification of the predentine becomes complete, the sudanophilia becomes less and less.

Observations have also been made on the long bones, and will be reported elsewhere. The epiphyseal cartilage contains a sudanophil material, which disappears during rickets and promptly reappears after vitamin D administration.

There is strong evidence for the view that a mucopolysaccharide, similar to chondroitin sulphuric acid, is involved in calcification (BOYD and NEUMAN, 1951; SOBEL and BURGER, 1954). It has always been difficult to explain why cartilage, with its high content of chondroitin sulphuric acid, does not calcify. If the present findings indicate that a specific type of mucopolysaccharide, only found at calcification sites, is concerned in the calcification mechanism, then the theory as a whole is considerably strengthened.

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A HISTOLOGICAL STAINING METHOD FOR SITES OF CALCIFICATION

All sections were stained with Sudan black after pyridine treatment, as described in the text. The tooth sections are all longitudinal sections from the labial side of the rat's upper incisor tooth.

e.o., enamel organ; e, organic enamel; d, dentine; p, predentine; od, odontoblasts; r.p., rachitic predentine; p.d., predentine formed after vitamin D dosage; c, calcification line; os, osteoblasts.

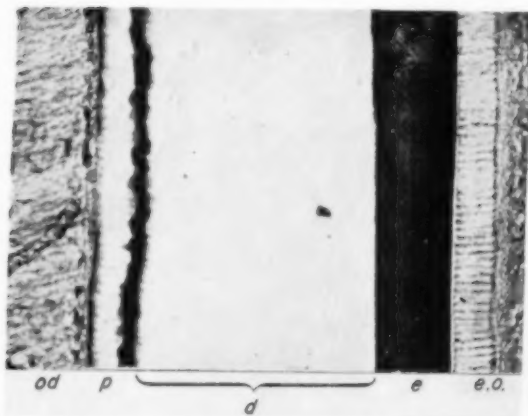


FIG. 1. Normal tooth. Note the intense staining line at the dentine-predentine junction, and also that the organic enamel stains more on the bottom where it is becoming acid-soluble. $\times 200$.

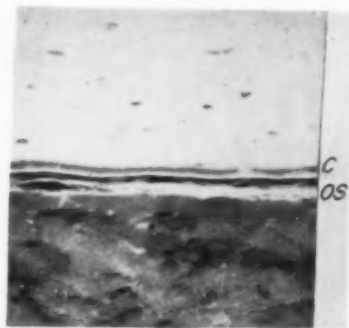


FIG. 2. Section of bone showing the calcification line. $\times 220$.

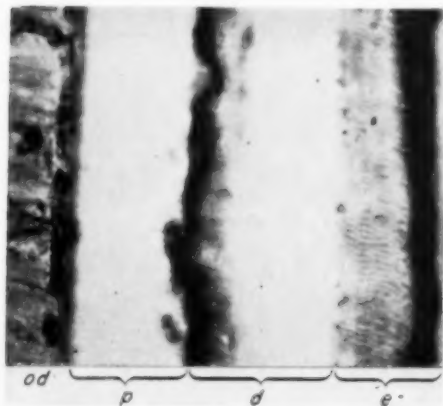


FIG. 3. Tooth from rachitic rat. Note the wide predentine, and the staining at the dentine-predentine junction. $\times 220$.

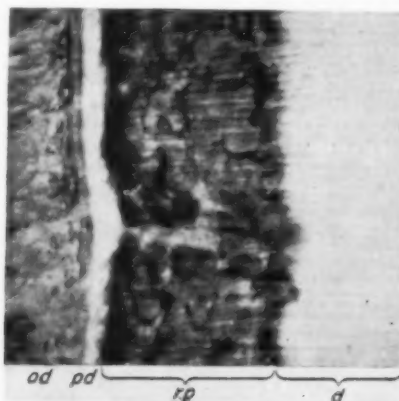


FIG. 4. Tooth from rachitic rat, 4 days after vitamin D dosage. Note the intense sudanophilia of the rachitic predentine, and the formation of new predentine of the proper width. $\times 220$.

All sections were stained with Sudan black after pyridine treatment, as described in the text. The tooth sections are all longitudinal sections from the labial side of the rat's upper incisor tooth.

e.o., enamel organ; e, organic enamel; d, dentine; p, predentine; od., odontoblasts; r.p., rachitic predentine; p.d. predentine formed after vitamin D dosage; c, calcification line; os, osteoblasts.

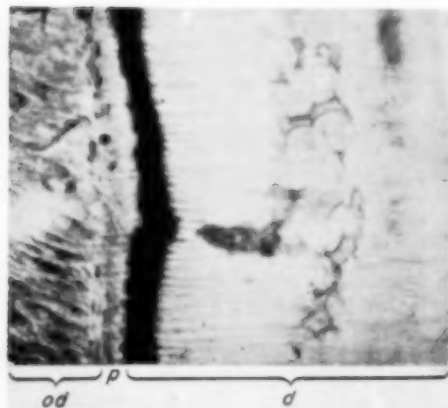


FIG. 5. Tooth from rachitic rat 8 days after vitamin D dosage. Note the intense staining line at the dentine-predentine junction, and also some residual sudanophilia of the dentine. $\times 220$.



FIG. 6. Section of bone from rachitic rat. The calcification line is at the edge of the osteoid seam. $\times 220$.

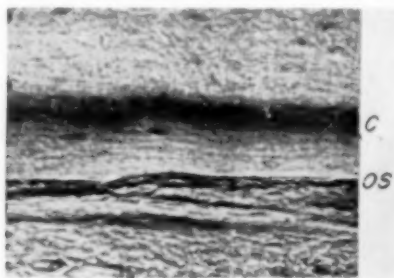


FIG. 7. Section of bone from rachitic rat 6 days after vitamin D dosage. The sudanophilic calcification line is spreading through the osteoid seam. $\times 220$.

PIGMENTATION OF THE ENAMEL OF ALBINO RAT INCISOR TEETH*†

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Abstract—The yellow pigment of the incisor tooth of the normal albino rat becomes visible at approximately the midpoint of the convex, enamel-covered surface. Pigmentation occurs in an area incisal to the region where full thickness of the enamel has been attained and after complete acid solubility (maturation) has occurred. The pigment is confined to the outermost zone of the enamel. This zone is relatively acid resistant as compared to the underlying unpigmented portion of enamel.

Prior to the appearance of pigment in the enamel, the overlying enamel organ contains yellow pigment granules. Concurrently with the deposition of pigment into the enamel the granules disappear from the ameloblasts.

When the pigment-containing part of the enamel organ is surgically destroyed no pigment is deposited in the underlying enamel but the enamel surface is not otherwise affected.

A primary cuticle is described as present over the entire surface of the enamel except for the proximal zone where enamel matrix is being deposited. The possible role of this cuticle in calcification (maturation) and sideration (pigmentation) is discussed.

ALMOST half a century ago ERDHEIM (1911) observed that experimental hypoparathyroidism caused loss of pigment and the appearance of an opaque enamel surface in rat incisors. Since that time there have been published numerous reports on enamel-pigmentation disturbances due to nutritional deficiencies, hormonal disturbances, and intoxications by various substances (see PINDBORG, 1947, and TORELL, 1955, for reviews of the literature).

STEIN and BOYLE (1941) stated that the yellow or deep-orange pigment present in the outer part of the enamel appears approximately midway (Fig. 2) from the formative to the incisal end of the rat incisor, i.e. in the region where the full width of the enamel has been formed and where calcification has progressed to a stage where all or almost all the enamel disappears upon decalcification (Figs. 3 and 4). Upon histologic observation yellow pigment in the form of granules is observed in ameloblasts and the cells of the stratum intermedium beginning at about the border between the proximal and middle thirds of the tooth (Figs. 8 and 10). The pigmented enamel layer becomes relatively resistant to the action of 5% nitric acid (Figs. 4

* All experimental procedures were performed at the Harvard School of Dental Medicine prior to 1944.

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and 5). Through decalcification of the stained part of the incisor, two membrane-like structures are demonstrable: (1) an outer, primary cuticle approximately $1\ \mu$ in thickness and (2) a yellow layer of enamel approximately $8\ \mu$ in thickness. Moreover, surgical destruction of the enamel organ in the proximal third of the tooth resulted in spots of uncoloured enamel observed upon subsequent eruption of the affected tooth structure (Fig. 14). Certain of our findings have been confirmed by others (BUTCHER, 1953; WEINMANN, 1943; PINDBORG, 1947, 1953).

The present report presents a detailed description of the material upon which our original abstract was based (STEIN and BOYLE, 1941).

MATERIAL AND METHODS

Incisors of thirty-eight, chiefly adult, rats have been examined. Most observations were made either on undecalcified cross and longitudinal sections cut with a 'lightning' disc rotating under water at high speed and subsequently ground by hand. In order to preserve the enamel organ *in situ* the specimens were fixed in 10% formalin. They were not allowed to become dry in any stage of preparation. For the same reason grinding by hand was performed at a slow rate. The ground sections were dehydrated, embedded in balsam and for the most part left unstained. Certain sections were decalcified, using either 5% or 2% aqueous solution of nitric acid or 5% sulphosalicylic acid, and the process of decalcification was observed microscopically. Corresponding parts of the tooth of the opposite jaw were decalcified, embedded, cut in serial sections (transverse and longitudinal) and mounted for microscopic examination. In other animals the enamel organ was removed from the underlying enamel, embedded and sectioned without decalcification. In still another group, the soft enamel matrix at the formative end of the tooth was removed together with the overlying enamel organ and sectioned without decalcification. Unstained sections as well as sections stained by haematoxylin and eosin, phosphotungstic acid and haematoxylin, Mallory's aniline blue connective tissue stain, Turnbull's and Berlin blue were examined. In a few instances the yellow outer layer of the enamel was isolated during short-time decalcification by means of 2% nitric acid under the dissecting microscope and then embedded and sectioned. Undecalcified sections of teeth were X-rayed and incinerated in an S. S. White electric oven designed for fusing porcelain.

In addition the enamel organs of twelve teeth were surgically damaged in various parts of the incisor in an endeavour to learn more about the function of the ameloblasts.

OBSERVATIONS

The ground sections prepared were approximately $100\ \mu$ in thickness. The enamel organ and the periodontal tissue between the tooth and the surrounding alveolar bone were usually preserved in approximately normal relations in the cross sections. When attempts were made to prepare longitudinal ground sections of the incisor tooth *in situ*, the enamel organ and overlying alveolar bone often separated from the tooth surface as the section thickness approached the goal of $100\ \mu$. The strength of attachment of the enamel organ to the enamel surface was found to vary in different

regions of the tooth. This is in accord with the observation of others (KALNINS, 1952) that in the constantly growing teeth of rodents, the junction between enamel organ and enamel matrix is unstable and vulnerable not only *post mortem*, but also *intra vitam*. It was difficult to maintain the connexion between the ameloblasts and the enamel surface during the removal of the surrounding bone in the region where enamel matrix was being deposited. The adherence between the ameloblasts and the enamel surface increases where the yellow pigment is present in the ameloblasts, and it is greatest before the yellow stain occurs in the enamel proper (Fig. 9).

* According to BOYLE and KALNINS (unpublished) the curvature of the rat upper incisor can be compared with the dial of a watch between 9 and 4 o'clock. From Fig. 1 we can see that the growth and maturation of enamel matrix occurs in the portion corresponding to 9-10.45; the tooth erupts from the alveolus (labial alveolar crest) at a point corresponding to 1.30; and it appears in the mouth cavity before 2.30.

In a longitudinal ground section of the upper incisor of an adult rat the yellow substance in the ameloblasts (Figs. 8, 9, and 10) may first be observed in the area corresponding to 10.45-11 (at approximately 7-9 mm from the formative end). Since the rate of growth of the upper incisor is approximately 2 mm a week, this corresponds to the fourth week after the beginning of enamel matrix formation of the particular ameloblast.

The area where the yellow pigment disappears from the ameloblasts and appears in the outermost layer of the enamel varies. In teeth measuring 25-27 mm in total length, this transition area was found to be 10-15 mm from the formative end. This takes place at the fifth to eighth week following initiation of enamel matrix formation of the particular cell, corresponding to 12-12.45. Within a half week (1 mm) this yellow substance disappears from the ameloblasts and appears in the fibrous part of enamel (Fig. 9).

The yellow stain shows a strongly positive reaction to Turnbull's blue and Berlin blue (Fig. 11). It tends to disappear from the cells during decalcification. The pigmented enamel layer becomes brick red upon incineration. The pigmented layer is relatively acid resistant. This property is shown when nitric acid is applied to ground sections. Whereas the non-pigmented portion of enamel becomes dissolved in a few minutes, the pigmented layer becomes dissolved within 3-4 hr (Figs. 4 and 5). After application of acid the whole yellow layer peels off. It is rather brittle and it is not easy to get a continuous sheet of it.

That formation of the pigmented layer occurs due to alteration of the outer zone of enamel but not due to the addition of a new layer (as in the case of cuticle) is established by measurements of cross sections. There is no measurable increase in thickness of the enamel due to the appearance of the pigmented layer. The pigmented layer is 6-8 μ in thickness and shows a pattern similar to the arrangement of the ends of enamel rods (Fig. 6). This pattern is caused by short projections from the inside of the layer. These projections become visible on the inner surface of the pigmented enamel when decalcification of the acid soluble portion of enamel is complete.

There is a colourless and structureless membrane, the enamel cuticle, between the enamel and the ameloblasts (Fig. 12). This cuticle appears as soon as the full

thickness of enamel matrix becomes established and it persists unchanged to the incisal edge. No change in the appearance of the cuticle could be noted following the passage of the yellow substance from ameloblasts into enamel. By the action of acid alone the pigmented layer of enamel and the cuticle do not always separate from each other distinctly. Sometimes this separation could be obtained by tapping slightly with the point of a dissecting needle against the coverglass during the process of decalcification. It was noted that the adherence of the cuticle to the enamel surface is stronger than to the ameloblasts. If the ameloblasts have become detached from the enamel during preparation of the specimen, the membrane remains in connexion with the enamel surface. However, it can then be separated from the enamel surface by decalcification. This separation takes place only in those regions where the enamel is acid soluble. Near the formative end a region is reached where the enamel is not as yet of full thickness and does not disappear under the action of acid. No cuticle is demonstrable in this region.

If the enamel organ is surgically destroyed in an area where the cuticle is present and where the pigment is still concentrated in the enamel organ (yet not deposited into enamel), a non-pigmented portion of the tooth eventually erupts into the mouth cavity. This is soon followed by normal yellow stained enamel (Fig. 14). The enamel surface contour is not affected.

DISCUSSION

It has been stated by other workers that when the full width of the enamel is complete, the last product of ameloblasts is the primary cuticle (NUCKOLLS, LEICESTER and DIENSTEIN, 1947); calcified enamel is readily soluble in dilute acid and leaves no remnants in sections of decalcified teeth (CHASE, 1940).

Our results show that, after the full thickness of enamel is formed and latter becomes acid soluble, a new activity of the ameloblasts is evident as an accumulation of pigment in their protoplasm. This material then passes through the cuticle and is deposited into the outer zone of the enamel.

A contrary opinion, that the enamel matrix is not fully formed nor fully calcified at the time pigmentation occurs, has been expressed by PINDBORG (1947).

According to this investigator pigmentation in rat incisors extends 3-4 mm further apically from about one-half of the total length of the lower tooth and 3-4 mm further apically from about two-thirds of the total length of the upper tooth. However, the pigmentation in these apical 3-4 mm does not adhere to the surface of the teeth but "can easily be wiped off". From this macroscopic observation he concluded "that the imbedding of the pigment must take place at a time when the enamel has not yet been fully formed".

On the basis of his histologic study (his Figs. 2-4), including the Prussian blue reaction, he believes that "in a particular place the iron leaves the ameloblasts and passes into the enamel matrix the outer part of which becomes bluish".

Since in our specimens the yellow pigment is firmly fixed in the outer layer of enamel we cannot confirm PINDBORG's observation that the pigmented layer could be

"easily wiped off". Apparently he mistook the pigmented enamel organ for what he terms "pigmented outer part of enamel matrix".

In PINDBORG's Fig. 2, it is obvious that the form of the ameloblasts and the papillary arrangement of the remainder of the enamel organ as reproduced is inconsistent with the appearance of these structures in an area where amelogenesis is still occurring. It is also noteworthy that the zone of unstained enamel immediately below the ameloblasts is the same width as the zone of iron reacting enamel shown in his Fig. 4. The remainder of the enamel has disappeared in his Fig. 4, indicating its acid solubility. PINDBORG mistakenly interprets the acid resistance of the iron-reacting pigmented enamel shown in his Fig. 4 as indicating that it is still in the enamel matrix stage.

A positive reaction to Turnbull's blue and Berlin blue confirms the suggestion also of other workers (DAM and GRANADOS, 1945) that this substance is an iron-rich pigment rather than a porphyrin, lipochrome or melanin. Moreover, the pigment of enamel becomes brick red upon incineration, further establishing the presence of iron. This pigment appears to harden, toughen and make acid resistant the outermost edge of rat incisor teeth.

Thus, the function of the enamel organ of the incisor tooth of the rat may be considered in three successive stages: (1) the deposition of an enamel matrix; (2) formation of the enamel cuticle and (3) the mineralization of the enamel matrix. The mineralization of the matrix occurs in two phases: (a) deposition of calcium salts (maturation) and (b) deposition of iron salts with resulting pigmentation (sideration*). Since the enamel cuticle is present during the phases of maturation and pigmentation, we suggest that this membrane may play a role in the transfer of minerals from the cells of the enamel organ to the enamel matrix and calcified enamel.

Acknowledgement—Credit is due to Mr. GEORGE LEMASTER, Department of Photography, Institute of Pathology, Western Reserve University, for taking certain of the photomicrographs.

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* Since the term "maturation" has been applied to the process of calcification of enamel, the subsequent process of iron pigmentation may be called "sideration".

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FIG. 1. The growth of enamel matrix occurs in the portion corresponding to 9-10.45 o'clock. The tooth erupts from the alveolus at 1.30 o'clock and it appears in the mouth cavity before 2.30 o'clock (BOYLE and KALNINS).

FIGS. 2 AND 3. The relation between stages of enamel development and enamel pigmentation in upper incisors.

FIG. 2. A longitudinal unstained ground section of the tooth of a young rat. The pigment appears in the enamel about mid-way from the formative to the incisal end of the tooth; it is at a point corresponding to 12.45 o'clock.

FIG. 3. Microradiogram. Enamel is shown as a narrow radiopaque band. The arrow shows the site of initial enamel pigmentation which corresponds to 12.45 o'clock.

PIGMENTATION OF THE ENAMEL OF ALBINO RAT INCISOR TEETH

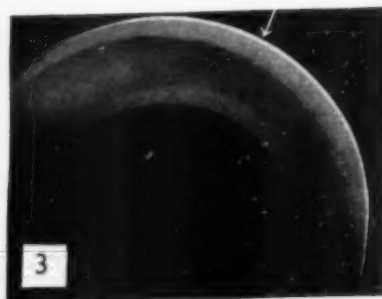
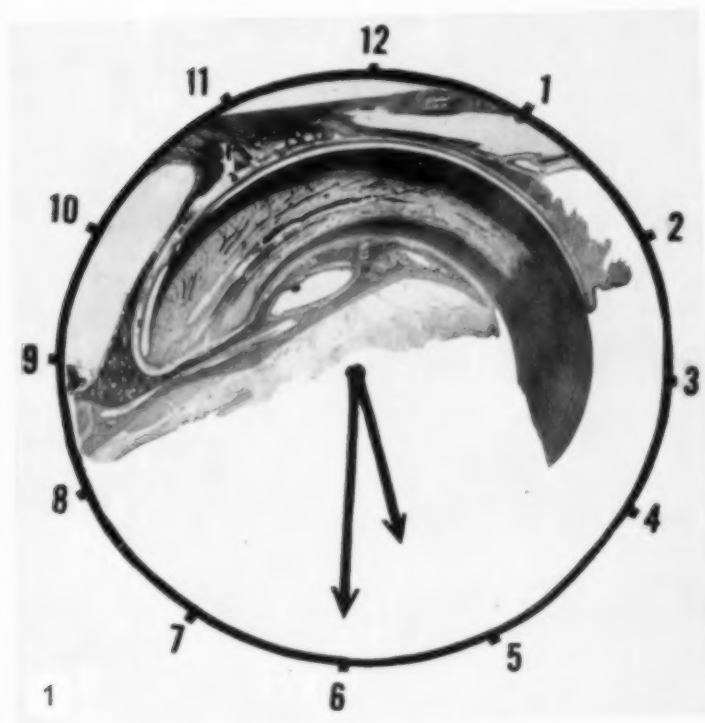
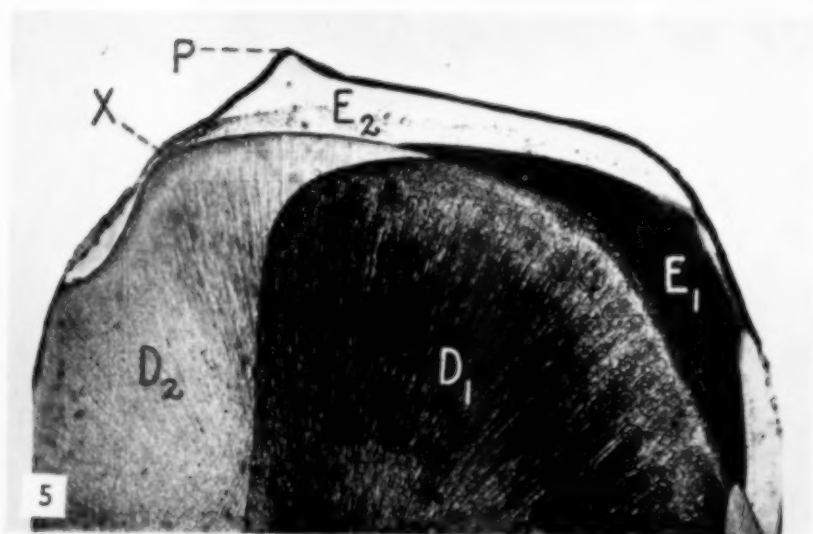
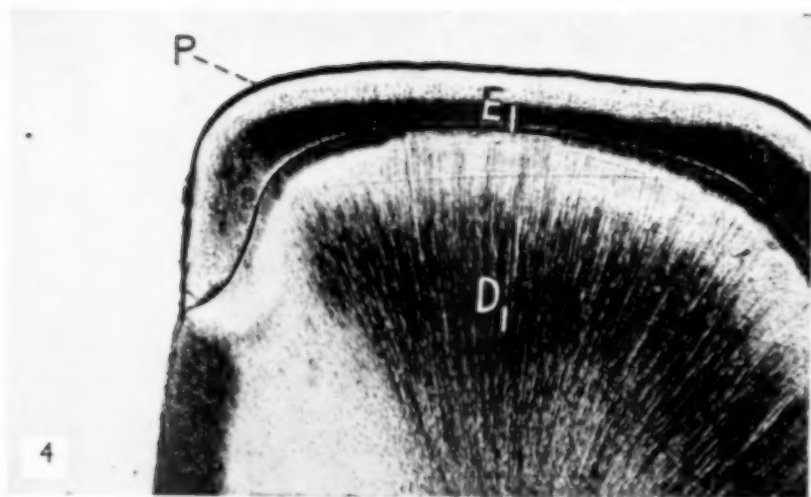


PLATE I



FIGS. 4 AND 5. These represent two views of an unstained ground cross section taken in the pigmented portion of an upper incisor. Fig. 4 shows the appearance before the addition of nitric acid and Fig. 5 during partial decalcification. Acid is drawn in from the left side so that enamel is further dissolved toward that side. In both figures the pigmented outer layer of enamel is shown in the form of a dark narrow band—P. Fig. 5 shows that under action of 2% nitric acid the inner non-pigmented layer of enamel becomes dissolved, whereas the outer pigmented layer (partially acid resistant) of enamel in the area indicated by X collapses toward the surface of dentine. E₁—non-pigmented enamel as yet undissolved by acid. E₂—space formerly occupied by the inner, non-pigmented layer of enamel. The variation between the dentine at D₁ and D₂ is caused by a gas bubble generated during decalcification of enamel.

FIG. 6. The honeycomb appearance of peeled-off sheet of pigmented layer of enamel seen from the under surface view. $\times 500$.

FIGS. 7, 8 AND 9. Show successive stages in the process of enamel pigmentation in an unstained ground section of an upper incisor in an adult rat.

In Fig. 7, upper incisor at a point corresponding to 11 o'clock, the enamel is already in full width and acid soluble. Here the ameloblasts are still without pigment. $\times 300$.

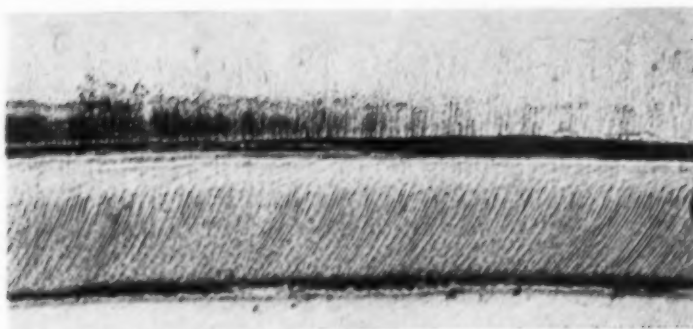
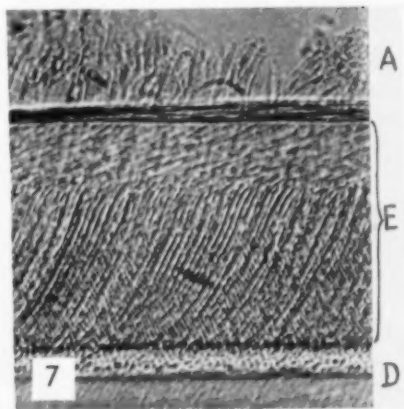
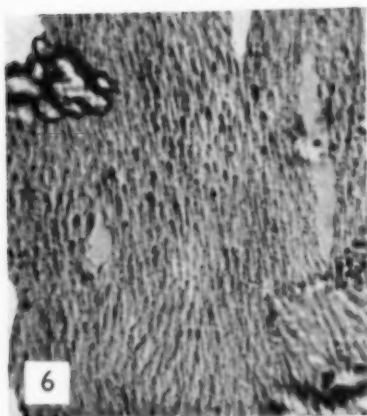
Fig. 8 shows that, at a point corresponding to 11.15 to 11.30 o'clock, accumulation of pigment in ameloblasts takes place and the cells appear yellow. $\times 300$.

Fig. 9 demonstrates a portion of the tooth corresponding to 12-12.15 o'clock. Here the pigment disappears from the ameloblasts and becomes visible in the outer layer of enamel. $\times 250$.

FIGS. 10 AND 11 show the enamel organ dissected away from the middle portion of the incisor, where the enamel is calcified (acid soluble) but still not pigmented. The specimen was not subjected to prior decalcification. $\times 600$. Fig. 10 shows that in the section stained with haematoxylin and eosin numerous fine granules of the yellow pigment appear in the cytoplasm of ameloblasts and in the stratum intermedium. In Fig. 11 a corresponding area is stained with Berlin blue, demonstrating that the granules give a reaction specific for iron.

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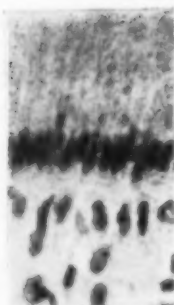
PIGMENTATION OF THE ENAMEL OF ALBINO RAT INCISOR TEETH



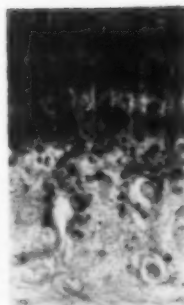
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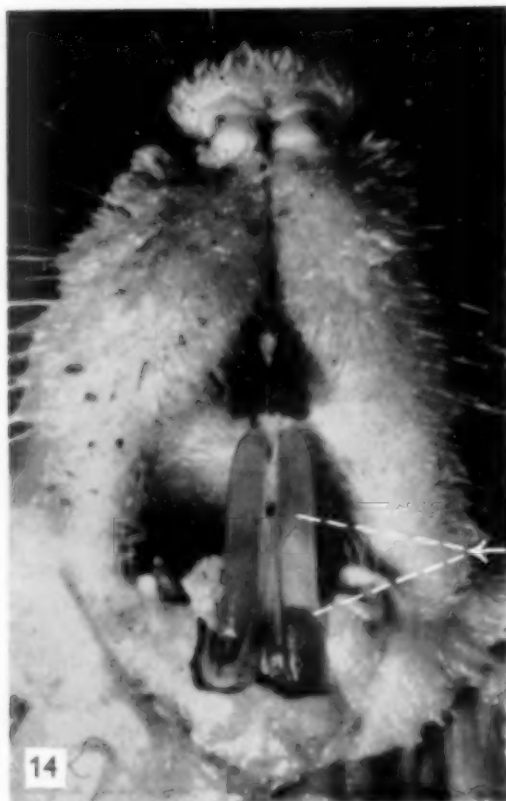
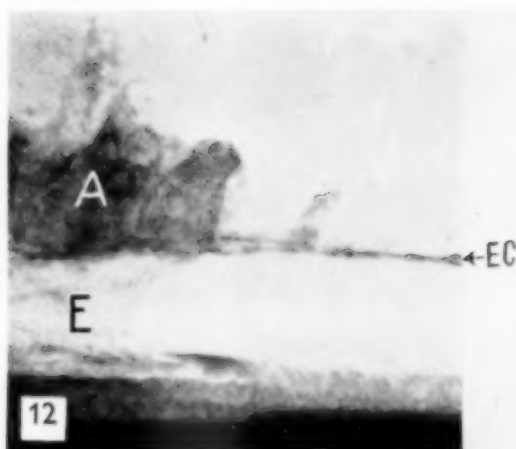


PLATE 4

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FIG. 12. Unstained longitudinal ground section showing the presence of enamel cuticle (EC) in the apical region of the incisor (at the point of 10.45 o'clock), where the enamel first becomes acid soluble. A—ameloblasts. E—enamel. $\times 300$.

FIG. 13. The sharp edge of the incisal end of a normally pigmented tooth. EP—the outer, pigmented, narrow layer of enamel. E—non-pigmented inner layer of enamel. Unstained ground section. $\times 500$.

FIG. 14. A non-pigmented spot (indicated by an arrow) in the enamel of a left lower incisor. This is the result of surgical destruction (by electrocauterization performed seven weeks previously) of the pigment containing enamel organ in the middle region of the tooth. Note that the colourless portion of enamel is followed by normally pigmented enamel. Other than the lack of colour, no defect in the enamel surface was detected. The rate of eruption of both incisors was equal.

MICRORADIOGRAPHIC OBSERVATIONS ON DEMINERALIZATION GRADIENTS IN THE PATHOGENESIS OF HARD-TISSUE DESTRUCTION

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Abstract—Hard-tissue changes accompanying dental caries, idiopathic erosion, traumatic abrasion, lacunar resorption and post-mortem excavation have been examined by means of microradiography. Carious lesions exhibit very extensive subsurface demineralization to a depth of up to 1000 μ , along preferential, incremental, interprismatic and intratubular pathways. Erosion is accompanied by a superficial demineralization gradient up to a maximum depth of 100 μ , characterized by a wide peripheral frontal attack rather than along preformed structural pathways. Abrasion, whether caused by toothbrush friction *in vivo* or *in vitro*, removes the organic and inorganic elements of the hard tissue simultaneously and *in toto*. Resorption of all hard tissues, be it in bone, cementum, dentine or enamel, follows a comparable histomorphological pattern, and all elements of the resorbable tissues disintegrate without any subsurface demineralization gradient beyond the depth of about 1 μ . Post-mortem destruction of calcified tissues takes the form of deeply penetrating canals, from which enamel is curiously spared, and which in dentine cut across the dentinal tubules and cause removal of inorganic and organic elements without any antecedent demineralization gradient detectable beyond the depth of about 1 μ .

No unified concepts have been established to account for either normal deposition or pathological withdrawal of tooth and bone minerals in the construction and destruction of the dental and skeletal hard tissues, enamel, dentine, cementum and bone, respectively.

The present study is concerned with a comparison between the destructive sequence of events, the relative extent of demineralization detected by microradiography of hard-tissue destruction. Notwithstanding similarities in construction, the following observations demonstrate distinct differences in the genesis of the principal types of destruction to which the hard tissues of teeth and bones are prone: caries, erosion, abrasion, resorption and post-mortem excavation, respectively.

MATERIAL AND METHODS

The present report is concerned with observations on human teeth, partly from the adult permanent dentition—teeth undergoing caries, erosion and abrasion; partly from the deciduous dentition—teeth undergoing physiological resorption and removed just prior to shedding time; and partly from exhumed skeletal material—teeth subject to post-mortem destruction. For comparative purposes, abrasion has also been studied as produced on teeth *in vitro*, resorption as it occurs in remodelling of alveolar bone.

The specimens were fixed in 70% ethyl alcohol, dehydrated and embedded in methyl methacrylate, sectioned with steel slotting saws, and further reduced in thickness by grinding according to procedures described in detail elsewhere (SOGNNAES, 1947). The sections were exposed to soft X-rays from a modified Norelco X-ray diffraction apparatus with a North American Philips X-ray transformer and horizontally placed four-window diffraction copper target tube with a beryllium window less than 1 mm and a thin coating of mica. In a vertically placed lead-coated camera the sections were placed at a distance of 25 cm from the X-ray source and exposed through direct contact with Eastman Kodak Spectroscopic 1 × 3 in. plates No. 649—G.H. Initially, the sections were exposed while relatively thick, over 100 μ , at 20 kV, 15 mA for 10 min. The thickness of the sections of selected specimens was reduced further and they were re-exposed to the X-rays at a lower kilovoltage (10–15). The rationale for this method has been described by ENGSTRÖM (1946). In essence, the procedure as here used may be said to reveal the calcium phosphate content of the hard tissue on a histochemical, volumetric basis; in other words, the density of calcification per unit volume of microscopically oriented regions of the hard tissue.

In addition to the microradiographic observations, a few preliminary hardness tests were made on selected areas by means of the new Kentron Knoop Vickers Micro Hardness Tester (Torsion Balance Company, New Jersey). Finally, for comparative purposes some specimens were decalcified and stained, mostly by routine methods, for identification of the cellular and vascular elements in the environment adjacent to the disintegrating hard tissues.

More specific treatment of these and other specimens will, when necessary, be added under the appropriate heading below. It should be emphasized that the resolution of the microradiographic method as here used is at best about 1 μ , and that other essential studies are in progress with a view to exploring the ultrastructural destructive surface changes extending to a depth of 1 μ or less during the incipient stages of hard-tissue destruction.

FINDINGS

(1) Caries

Two types of carious lesions were studied: (a) incipient enamel caries in which the surface contour of the tooth appeared unaltered, albeit of abnormal opacity, white and chalky, as grossly described elsewhere (SOGNNAES, 1940), (b) deeply undermining enamel caries extending into the underlying dentine. Special care was taken to section the central portion of the lesions.

Microradiographs of ground sections from carious teeth showed a demineralization front to a depth of 100–1000 μ (Figs. 1–5). In the enamel the destruction followed primarily the pathway of the incremental pattern (Retzius lines) and the interprismatic regions. The superficial layer retained relative high microdensity even though considerable demineralization occurred in the depth of the lesion (Figs. 1, 2). In Fig. 3, where enamel shows demineralization through the full depth of approximately 1000 μ , the intermediate, high-density layer may represent redeposition of

TABLE 1. HARDNESS TESTS SUPERIMPOSED ON MICRORADIOGRAPHY OF CARIOUS ENAMEL AND DENTINE

Location on carious tooth section*	Relative gradients in microradiographic density	Micro-hardness tests		
		No. of readings	Knoop numbers	
			Range	Average
Enamel				
A—subsurface	—	4	8-17	12
B—central zone	++	3	157-240	190
C—inner zone	+	1	139	139
Intact enamel	++++	3	317-317	317
Dentine				
D—subsurface	+	6	38-49	43
E—central zone	++	3	47-57	52
F—inner zone	+++	3	57-67	63
Intact dentine	++++	7	70-85	75

* For histomorphological orientation see Fig. 3 left.

dissolved minerals escaping from the deeper layers of demineralizing enamel and dentine. The resulting softening of the tooth substance is reflected in the micro-hardness (Table 1, Fig. 3, A-C) and in the abnormal stainability of the tooth substance (Fig. 3, right), including the intermediate "recalcified" enamel layer.

In acute dentine caries the demineralization similarly extended to a considerable depth beyond areas of microscopic breakdown of the organic matrix. At an early stage the low density areas appeared to proceed partly by way of the incremental zones (Fig. 4) and along the dentinal tubules so as to make them more apparent than in the normal underlying dentine (Figs. 4 and 5).

Correlating these changes in microradiographic density with previous observations on histochemistry (SOGNAES and WISLOCKI, 1950), it would appear that caries involves removal of the inorganic components to a considerable depth along the incremental, interprismatic and intratubular pathways, prior to extensive bacterial invasion and destruction of the organic matrix.

(2) Erosion

Textbooks on the pathology of the teeth do not appear to include any definitive description of the histopathology of erosion. "The most common form of erosion," according to BOYLE's 1949 edition of Kronfeld's text, "consists of wedge-shaped defects with sharply outlined borders; they are deeply cut into the tooth structure, and their floor is clean, hard and smooth in appearance, altogether different from caries." There is no evidence that this statement is based on histological examination, for it merely repeats the classical gross appearance to the naked eye as described before the turn of the century.

It is true that, in contrast to caries, dental erosion exhibits a relatively smooth and clean-looking surface to the naked eye. Nevertheless, we have found histologically that such lesions, the typical wedge-shaped variety, are not necessarily

devoid of superficial deposits of salivary and bacterial origin. Microradiographic examination of the lesions indicates that in the acute stage there is an external zone of demineralization which may extend to a depth of up to 100 μ (Figs. 6, 7, 8) prior to disintegration of the organic framework, a depth barely short of the resolving power of the human eye. Hence, no opacity is seen; the lesion appears "clean, hard and smooth". Erosion differs from caries by the fact that the destruction occurs by a frontal attack on the intertubular matrix rather than penetration along the dentinal tubules.

Our microradiographs indicate that, while the demineralization can extend to a maximum depth of 100 μ , such a gradient varies down to almost nothing in certain parts and stages of the lesions. Furthermore, some lesions, presumably chronic, appear slightly hyper-calcified along the surface (Fig. 6) as though some minerals may have become redeposited, possibly from saliva. The demineralization gradient, instead of being gradual (Fig. 6), may be layered, one superficial disintegrating zone, one intermediate zone of considerable demineralization and one deeper zone of lesser demineralization (Fig. 7). The dentinal tubules do not appear distended as in caries, but are about equally well seen in the demineralized zone as in the underlying dentine (Fig. 7). Occasionally, a lesion is found with irregular surface, possibly a combination of erosion with some more acute destruction, yet the demineralization gradient is very evenly distributed along a superficial zone of 100 μ or less (Fig. 8).

In decalcified sections we have noted, furthermore, three organic deposits in juxtaposition to the surface of these "eroded" areas: (a) a thin film which stains green with toluidine blue, possibly of protective significance; (b) a wider zone of metachromatic amorphous material, presumably salivary mucin; and occasionally (c) a zone of bacterial plaque. Where the first mentioned film exhibits discontinuity so that the mucous and microbial layer comes in direct contact with the tooth substance, there appears to be a frontal demineralization along the tooth surface.

Despite this superficial demineralization, erosion is not accompanied by bacterial invasion of the dentinal tubules. Furthermore the staining properties of the demineralized area, when examined in completely decalcified sections, do not appear markedly different from the adjacent dentine unaffected by the erosion. On the strength of these preliminary observations, it appears that mineral withdrawal is a primary mechanism in erosion, that this event precedes the loss of the organic matrix and occurs by a frontal superficial attack rather than along the preformed pathways as in caries. Whether this demineralization is due to local accumulation of acid or some sequestering agent, such as citrate, that may form a complex with the mineral through a process of chelation, is not known. Neither is it known whether or not the organic matrix, once it has been exposed to a certain depth, becomes removed through mechanical, bacterial, or biochemical means.

(3) Abrasion

Aside from the peculiarity in location of dental erosion (we have seen circumferential wedges producing an hour-glass appearance of lower anterior teeth), it is not easy clinically to distinguish idiopathic erosion from simple abrasion caused by mechanical friction.

In order to compare *in vivo* lesions with experimental wear and tear, several teeth were exposed to artificial abrasion *in vitro* in a tooth-brushing machine. A medium nylon bristled brush (Park Avenue brand) was trimmed to one row of tufts and mounted in a brushing machine. With a slurry containing 33% of anhydrous dicalcium phosphate abrasive, the brush was moved against the tooth surface at 200 strokes per minute with 150 g brush pressure. After a total of 5000 strokes a clearly visible U-shaped defect extended into the dentine. At this point the specimens were prepared for plastic-embedded ground sections as described above. Other teeth which had similarly appearing defects produced *in vivo* were sectioned for comparative microradiography.

It seemed possible at the outset that even tooth-brushing with a suspension of toothpaste might cause some superficial leaching out of minerals below the surface. This possibility could be ruled out by careful examination of teeth which exhibited deep, narrow, U-shaped defects and whose history and location were compatible with a clear-cut abrasion. The surface of these abrasion lesions was very smooth by all criteria from visual to microscopic examination. The microradiographic and other landmarks were identical to those which could be produced experimentally *in vitro* by the friction of tooth-brushing.

A typical natural lesion with a location and history suggestive of toothbrush abrasion is shown in Fig. 9, indicating (by contrast with Figs. 6, 7, 8) that the surface of the lesion is sharply outlined even at the microscopic level with no demineralization gradient visible in microradiographs. On the contrary, there is the suggestion of a slight secondary deposition of mineral matter along the surface, possibly from either the saliva or toothpaste agent. When similar lesions were produced experimentally by the tooth-brushing machine, a very similar condition can be shown—a sharply outlined U-shaped lesion with no evidence of drainage of minerals below the surface (Fig. 10). This similarity of the appearance *in vivo* and *in vitro* suggests that a bona fide mechanical abrasion can be produced in the teeth, unaccompanied by microscopically demonstrable leaching out of minerals below the surface; rather a virtually simultaneous removal of organic and inorganic matter, distinctly different from caries and erosion.

(4) Resorption

All human hard tissues, bone, cementum, dentine and even enamel, are prone to resorption: in connexion with the continuous physiological remodelling of the skeleton, especially trabecular bone; in the normal shedding mechanism involving the deciduous teeth; in root resorption associated with ageing and orthodontic tooth movement; in idiopathic tooth resorption, either from the pulpal or periodontal surface; in metabolic disturbances, such as hyperparathyroidism; and in the breakdown of alveolar bone during periodontal disease.

In teeth and bones alike, resorption occurs adjacent to cells situated in characteristic lacunae, but it is not established in these areas of lacunar resorption to what depth—if indeed at all—the inorganic components of teeth and bones are removed prior to the removal of the organic portion. On the one hand, it has been suggested that the organic fraction may be the site of primary changes adjacent to the osteoclasts. On the other hand, there have been speculations regarding chelation to account for the mineral withdrawal.

In addition to the microradiographic evaluation of the resorption sites in ground sections, one additional procedure was utilized in order to compare the relationship between the hard tissue resorption surface and the adjacent cells. To obtain thin undistorted paraffin sections presents special difficulties in the case of mature enamel, and to our knowledge no one has illustrated the histological relationship between the organic framework of mature enamel and adjacent cells located in lacunar resorption sites. Occasionally a shedding deciduous tooth is not uniformly resorbed, in which case the tooth may be retained on one side of the root while resorption continues through the dentine and into the enamel on the other side. Such specimens have been searched for with a view to recovering the adjacent cells in proper juxtaposition to the resorbing enamel. Special precautions had to be taken in order to preserve the matrix of such mature enamel which had outlived its full functional life span.

Following removal and a few days' submersion in 10% formalin, the teeth were prepared for paraffin sectioning by means of a decalcification procedure which may be referred to as a low-temperature "fix-as-you-go" technique. Each tooth was inserted separately in a small formalin-filled celloidin bag (made by coating the wall of a 0.5 in. glass tube with a 2% solution of celloidin), placed in an icebox at 1°C in a beaker containing the following solution: trichloroacetic acid (5 g) and potassium dichromate (2.5 g) dissolved in distilled water (100 ml) to which was added formaldehyde (10 ml) serving the dual purpose of decalcification and fixation. The celloidin bags served three purposes, the need for all of which was evident from a previous study on this problem (SOGNNAES, 1948): first, to slow down the initial penetration of the acid; second, to immobilize the specimen; and third, to keep it continuously surrounded by fluid when transferring the specimen from one solution to the other. During a week's submersion in the above-mentioned solution, the organic elements of the enamel were slowly exposed and thus simultaneously decalcified, fixed and hardened. The specimens, still contained in the separate celloidin bags, were then transferred to a 5% aqueous solution of trichloroacetic acid for an additional length of time not exceeding 2 weeks.

Next the specimens, still in the celloidin bags, were submerged in cold water for several washings, and carried carefully through increasing concentrations of ethyl alcohol to butyl alcohol. From this liquid, which is miscible with paraffin, the dehydrated specimens could be lifted into paraffin by means of a small spoon which had been made for that specific purpose. In this manner it was possible to keep the delicate enamel framework continuously in a fluid medium in order to prevent its collapse, tearing or loss. Having thus carefully transferred the specimens to paraffin, sections were cut in routine fashion, de-paraffinized, and treated with haematoxylin-eosin, azan and other stains.

For comparison were included observations of decalcified sections of alveolar bone resorption as seen in the reconstruction of the jaw bone in normal rhesus monkeys. These specimens were prepared as routine paraffin sections, using the formic acid-sodium citrate decalcification technique of Morse (1945).

In order to shed light on the surface changes accompanying resorption, it is necessary to examine sections of different thickness and orientation. Within thicker sections of 100 μ or more, one is liable to find overlapping of obliquely cut, concave lacunae giving the false impression of demineralization gradients. However, when sections are reduced in thickness to 30-50 μ through the centre of the lacunae, true profiles of the individual resorption cavities can be obtained. Under these circumstances we failed to note a gradient in demineralization comparable to that noted in the case of caries and erosion. In other words, where the section had gone through the middle of a lacunar surface, there appeared to be no detectable mineral withdrawal within the one micron accuracy of this method, but a sharp edge of the mineralized hard tissue surface. This held true of low and high density regions of alveolar bone (Fig. 11), as well as resorbing dentine of shedding teeth (Fig. 12). It is interesting to note that the cementum of the tooth root, despite its close proximity to the resorbing alveolar bone, may be completely protected from resorption (Fig. 11). This, however, is not always true. Sporadic lacunae of cementum resorption increase

with age (GUSTAFSON, 1950), and can become extensive under excessive trauma, in rapid orthodontic tooth movement, in the presence of highly vascularized pathological granulation tissue and, obviously, during the normal shedding of deciduous teeth.

During the physiological shedding of deciduous teeth, the resorption of the roots of the teeth is morphologically identical to the osteoclastic resorption of bone undergoing cellular remodelling in response to functional or metabolic influences. Occasionally, the resorption of shedding teeth may extend to the enamel. Fig. 13 shows a decalcified section of enamel from such a deciduous tooth from a 13 year old girl. Because of uneven resorption, this tooth did not become shed until resorption had proceeded into the enamel on one side of the tooth. The enamel matrix has been retained and shows Howship's lacunae, some but not all of which contain centrally cut giant cells. The enamel has retained its delicate network of interprismatic organic matter and, running vertically through the centre, a pronounced row of denser regions of the prism sheets, corresponding to the neonatal line separating prenatal and postnatal enamel (right).

This type of enamel resorption seems very similar to resorption of bone as shown in a section of the alveolar bone of a rhesus monkey (Fig. 14). In this decalcified section, the giant cells situated in Howship's lacunae are seen in near proximity to adjacent blood-vessels, and one of these cells (upper right) appears as though situated within a blood-vessel.

The above results indicate a close correspondence in microradiographic and histomorphological appearance between bone and tooth surfaces undergoing resorption, both from the point of view of the typical lacunar resorption sites and the adjacent cellular elements. In view of the distinct differences which exist between the nature of these several hard tissues, it would appear difficult to single out one particular feature typical of the bone and tooth structure which can be invoked as inducer of resorption. Resorbability is not necessarily dependent upon the range of calcification which can cover the full spectrum of nearly 100 per cent (mature enamel) down to uncalcified (predentine) without *a priori* promoting or resisting resorption, or upon

TABLE 2. INTERCELLULAR VARIATIONS IN RESORBABLE TISSUES

Type of tissue	Relative degree of calcification	Abundance of amorphous ground substance	Fibrous framework
Predentine	—	—	Collagen +++++
Bone	++	++	Collagen ++
Primary cementum	++	+++	Collagen +++
Secondary cementum	+++	++	Collagen ++
Dentine	++++	++	Collagen ++
Enamel	+++++	+	"Keratin" +

the amount and nature of the amorphous and fibrous organic elements (Table 2). Nor can the cells located in the lacunar resorption sites be attributed to antecedent hard tissue cells, since no interstitial cells are present in primary cementum, dentine

and enamel, notwithstanding the co-existence of typical giant cells in the resorption lacunae in all of these tissues (Table 3). At the moment, therefore, it would appear (a) that resorbability is not dependent upon any fixed proportion of organic-inorganic

TABLE 3. CELLULAR VARIATIONS IN RESORBABLE TISSUES

Type of tissue	Adjacent cells	Interstitial cells
Bone	Osteoblasts	Osteocytes
Secondary cementum	Cementoblasts	Cementocytes
Primary cementum	Cementoblasts	None
Predentine	Odontoblasts	None
Dentine	None	None
Enamel	None	None

interstitial hard-tissue elements, be they crystalline, fibrous, amorphous or cellular; (b) that the typical giant cells located in the lacunar resorption sites do not necessarily originate from genuine hard-tissue cells, but may be brought into juxtaposition to the resorbable surfaces from the adjacent vascular environment.

(5) *Post-mortem excavations*

HOPEWELL-SMITH (1918), in his textbook on histopathology of the teeth, describes a disease which he calls "fungoid excavations". He illustrates the condition by two cases in which microscopic sections revealed numerous large canals criss-crossing the dentine. Whereas HOPEWELL-SMITH suggests that this type of excavating canal represents a new and extremely rare type of pathology occurring during life, it is significant that a footnote indicates that the two rare specimens were observed in exhumed teeth in which we have demonstrated that this particular type of destruction, on the contrary, is extremely common (SOGNNAES, 1955b).

We have studied exhumed human teeth which date back to eleven periods of history, paleolithic to recent, and originate from six geographic locations, Palestine, Egypt, Greece, Iceland, Norway and Central America. From all sources a total of 209 teeth were obtained and several ground sections were prepared of each tooth, altogether about 800 sections. All of these were examined in transmitted light. In addition, selected specimens were prepared for microradiography and some fragmented teeth for decalcified paraffin sections.

The root surface of many of the exhumed teeth presented a dry, porous and dull appearance. Unlike anything observed in freshly extracted specimens, it was found that abnormal canals either entered the teeth from the inside through the dentine or penetrated the cementum-covered roots from the outside. One type was characterized by long and narrow canals 2-10 μ thick. Another type showed a tendency to form several branches, 15-25 μ thick. The largest canals, and hence the most destructive ones, sometimes reached a diameter of from 50 to 100 μ and were easily identified by their ampula-shaped widenings.

Two of the most significant characteristics of these canals were, first, their width which exceeded that of the normally present dentinal tubules, and second, the fact that the canals penetrated in all directions and across the intertubular matrix rather than along the dentinal tubules (Figs. 15, 16, 17). Histologically, there were distinctive differences between the post-mortem penetration and the *intra vitam* type of invasion characteristic of caries. Unlike caries, which usually follows definite pathways in a consistent relationship to the morphological pattern of the teeth, the post-mortem canals were very irregular, running across rather than along the direction of the dentinal tubules. Another histological characteristic, suggesting post-mortem destruction, was the absence of vital response in the neighbourhood of the "cavities". No evidence of secondary or transparent dentine, which are familiar reactions to *intra vitam* lesions of the teeth, could be observed in a logical proximity to the type of destruction just described.

In microradiography this type of post-mortem decomposition shows destruction of both cementum and dentine with complete disintegration of the calcified matrix without any gradient in demineralization (Fig. 15). Ground sections examined in transmitted light indicate, in contrast to caries, that the destruction is haphazard without any regard to the incremental pattern or dentinal tubules (Figs. 16, 17).

The enamel, which has a similar inorganic component (apatite) and is the most acid-soluble part of the teeth, is protected from this type of destruction. This process is not due to a generally acid environment surrounding the whole jaws and teeth, but must be due to destructive saprophytic agents seeking the collagenous matrix of the mesenchymal hard tissues.

Until similar canals are demonstrated in sections of freshly extracted teeth, it must be concluded that we are dealing with a condition which has not been proved to occur during life but is very common after death.

DISCUSSION

On a comparative basis and within the limitations of the microradiographic technique, the present study, as summarized in Fig. 18, emphasizes significant differences rather than similarities between the several situations in which the hard

ENVIRONMENTAL AGENTS AND DEMINERALIZATION GRADIENTS
IN THE PATHOGENESIS OF HARD TISSUE DESTRUCTION

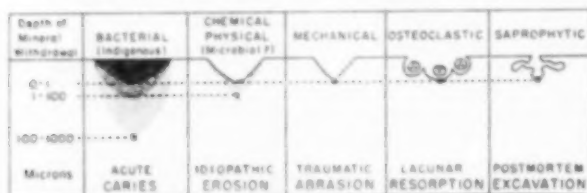


FIG. 18. Diagram summarizing the results of the microradiographic analysis indicating the relative extent of demineralization gradients, decreasing significantly from caries and erosion to resorption and post-mortem destruction of the calcified tissues.

tissues break down. Confronted, on the one hand, with an external oral environment conducive to caries, the enamel and dentine can be subject to a very deep demineralization, to a depth of as much as 1000 μ , before grossly visible collapse of the tissues results in full-fledged cavity formation. Confronted, on the other hand, with an internal environment conducive to hard-tissue destruction, the opposite end of the spectrum is observed insofar as full-fledged lacunar resorption cavities can be demonstrated, be they in bones or teeth, seemingly without any preceding demineralization within the resolving power of the X-ray microscopy, and hence presumably less than 1 μ . The same appears to hold true for post-mortem destruction caused by saprophytic excavation. Between these destructive extremes, the so-called idiopathic erosion of the teeth occupies an intermediate position in which can be demonstrated a zone of microscopically detectable demineralization which may range from minimal to a maximum of 100 μ , barely short of the visualizing power of the human eye. If this softened microscopic surface layer is removed by mechanical friction the eroded

surface cannot be readily distinguished—even by microradiographic examination—from uncomplicated abrasion in which we have found no evidence of subsurface mineral withdrawal prior to total tissue destruction.

To account for these microradiographic findings, it must be assumed that the agents responsible for incipient carious destruction—unlike those in erosion and resorption—penetrate preferential pathways far beyond the surface. The relatively higher resistance to dissolution of the peripheral enamel zone is in keeping with other studies using polarized light and radiography (DARLING, 1956; GUZMAN, BRUDEVOLD and MERMAGEN, 1957) and may well be accounted for by a lesser solubility imparted by the relatively much higher concentration of fluoride in the surface than in the deeper layers of enamel (BRUDEVOLD, GARDNER and SMITH, 1956).

The nature of the agents responsible for the carious dissolution of enamel and dentine is not known. Recent advances in experimental caries research (SOGNNAES, 1955a) have indicated the complexity of this disease by invoking genetic, developmental, nutritional, hormonal and salivary factors, in addition to the now well-established environmental influences: indigenous oral micro-organisms coupled with an oral food substrate of refined carbohydrates, notably sugar, in a form which is retained around the teeth for prolonged periods of time. However, the extensive demineralization in depth along preformed pathways beyond the direct frontal microbial attack remains to be explained in terms of the dissolving agents on the one side, and the ultrastructural changes in the organic and inorganic tissue components on the other. Superficially, the initial histomorphological and histochemical changes (SOGNNAES and WISLOCKI, 1950) appear to cause a reversion of the tooth structure to a more "immature appearance", with a more pronounced pattern of incremental lines and cross-striations of the prisms—both well-known characteristics of early enamel caries which can readily be duplicated by exposure of enamel to acid *in vitro*.

In erosion, the aetiological involvement of oral micro-organisms has neither been demonstrated nor ruled out. The limited demineralization front would have to be explained either by a type of extraneous oral flora, agent or substrate different from that prevailing in caries, or by a tissue response to similar influences modified by some peculiarities of the tooth substance which prevent deep preferential penetration through the usual structural pathways. This question is being explored and will be discussed in greater detail in a later report. Meanwhile, the most significant single study of a chemical agent potentially involved in erosion is that of ZIPKIN and McCLURE (1949), who found a statistically significant positive correlation between salivary citrate content and the presence of dental erosion in adult man.

In resorption, recent work has indicated, at least by association, a localized accumulation of citrate in regions of active bone destruction. On general principles, McLEAN and URIST (1955) have favoured a sequestration or chelation, independent of local acidity, as the most probable mechanism for removal of bone salts in biological resorption. NEUMAN and NEUMAN (1958) cite observations *in vitro* favouring this concept and conclude, on the basis of preliminary experimental results *in vivo*, that the citrate content of the fluids in contact with resorbing bone may be

twice that in the general circulation. In our own laboratory, the most dramatic experimental bone resorption has been achieved by oxygenation (hyperoxia) of bone fragments (mouse calvaria) in tissue culture (GOLDHABER, 1958). Furthermore, it has been possible by the use of this new experimental resorption system to demonstrate that the resorptive process is accompanied not only by the formation of Howship-like lacunae housing newly differentiated multi-nucleated giant cells, but by elaboration of citric acid *de novo*, at a concentration very far beyond what could be accounted for by the bone-borne citrate removed with the dissolved calcium and phosphorus from the resorbing hard tissue (KENNY, DRASKOSZY and GOLDHABER, 1959).

There is still considerable uncertainty with respect to the origin of the giant cells and the precise sequence of events in resorption. McLEAN and URIST (1955) share the view of HANCOX (1949) that the osteoclasts are more than coincidentally involved and that these cells commonly arise by the fusion of a number of osteoblasts, and may also incorporate osteocytes liberated by the bone being resorbed. However, taking into account the fact that all of the hard tissues are prone to lacunar resorption, one cannot point to any comparable cellular elements or to any consistent proportion of inorganic and organic ingredients within bones and teeth, *per se*, to explain the origin of the multinucleated osteoclasts on the one hand, and the susceptibility to resorption on the other. It is suggested, therefore, that changes in the vascular and cellular environment adjacent to the disintegrating hard-tissue surfaces must be explored for factors that induce the virtually simultaneous removal of organic and inorganic elements of these tissues in the histopathogenesis of the resorption process.

One may theorize—by the simplest kind of reasoning—that, during biological breakdown of calcified tissues, the component parts are withdrawn in the reversed order of their deposition, that pathogenesis reflects the opposite events of histogenesis, the mirror image as it were. Development of the osseous and dental hard tissues commences with elaboration of the fibrous and amorphous elements of the organic matrix, whereas the inorganic crystals are the last components to appear. To liken this biological sequence of events to the stacking of a chemical warehouse may not seem inappropriate in view of modern concepts on the dynamic remodelling of bones and teeth (McLEAN and URIST, 1955; NEUMAN and NEUMAN, 1958; SOGNAES, 1955c, 1957). Furthermore, such an analogy in the present context would lead to the hypothetical conclusion that the last ingredients to be stacked and stored away when bones and teeth undergo construction (e.g. the inorganic building blocks), would be the most accessible and presumably the first ones to be attacked and carried away when these calcified tissues undergo destruction.

Currently, studies are in progress at the ultrastructural level to test this concept by electron microscopic examination of the superficial changes occurring to a depth of less than $1\ \mu$ during the incipient breakdown of mineralized tissues. Preliminary observations of thin sections cut with a diamond knife suggest that, at the sub-microscopic level of organization, there is indeed even in resorption a narrow zone of inorganic crystal destruction, to a depth of a fraction of a micron, leading to a biological unmasking of the organic framework. A more detailed discussion is beyond the scope of this paper and must be deferred until additional information is available.

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FIGS. 1-5. Microradiographic density of carious enamel and dentine (ground sections).

FIG. 1. An early carious lesion on the smooth surface of a molar showing demineralization to a depth of several hundred microns, primarily along the pathway of the incremental pattern of enamel. Despite the extensive demineralization in depth of the lesion, the superficial layer has retained relative high microradiographic density. In the centre of the lesion is a narrow band of intermediate density, possibly a zone of re-precipitated minerals. $\times 90$.

FIG. 2. An incipient lesion of occlusal fissure caries in a premolar showing the presence of a thin superficial dense layer despite subsurface demineralization with a depth of several hundred microns. $\times 40$.

FIG. 3. Caries of enamel and dentine, comparing microradiographic density and microhardness (left) with transmitted light appearance of stained ground section (right). Within the enamel lesion there is an intermediate zone of relatively high mineralization followed by extensive demineralization in the deeper zones of the enamel, up to a depth of $1000\ \mu$, and undermining dissolution of the dentine matrix. Letters A to F refer to the microhardness tests shown in Table I. On the right is the same section stained with toluidine blue and showing an abnormally high stainability of the intermediate high density enamel zone marked "B" on left. $\times 30$.

FIG. 4. Caries of the dentine. Demineralization has occurred to a depth of several hundred microns. The low density areas appear to proceed by way of the incremental zones and along the dentinal tubules so as to make them more apparent than in normal dentine. $\times 90$.

FIG. 5. Caries of the dentine, with pronounced loss of minerals to a depth of several hundred microns. In the deeper zones, the walls of the dentinal tubules appear distended because of demineralization of the adjacent matrix. $\times 90$.

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MICORADIOGRAPHIC OBSERVATIONS ON DEMINERALIZATION GRADIENTS

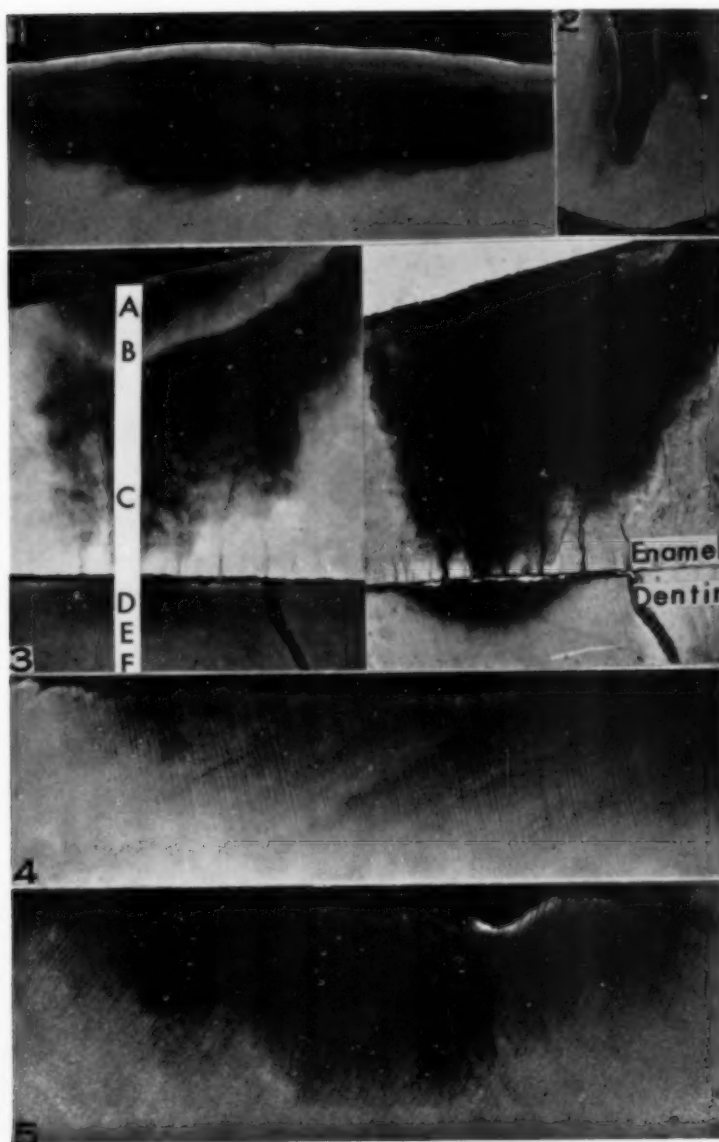


PLATE 1

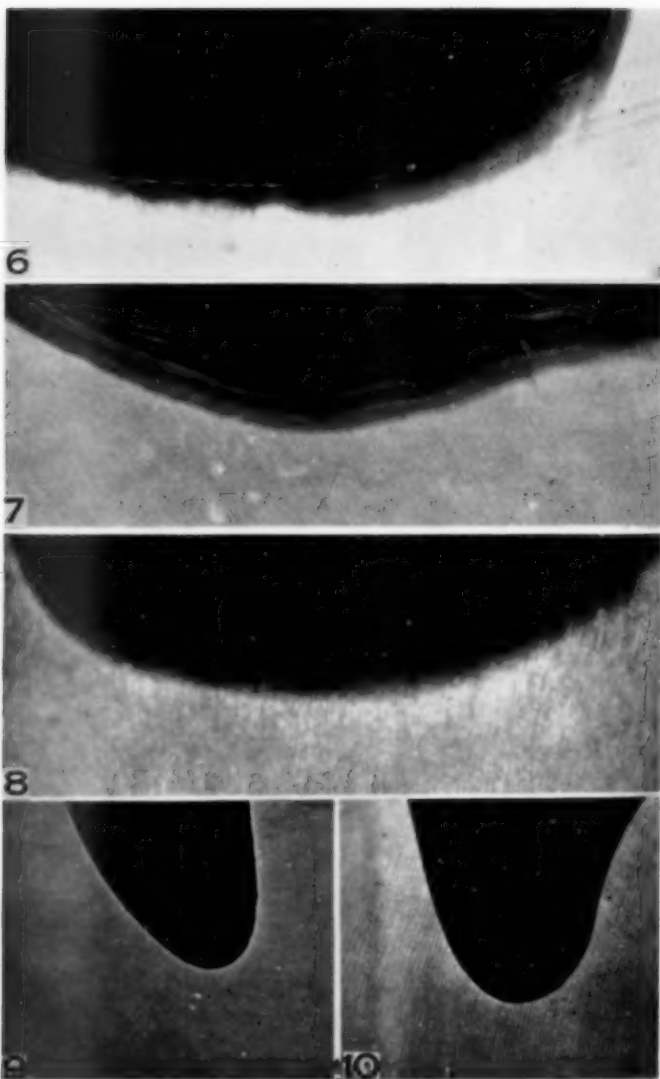


PLATE 2

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FIGS. 6-10. Dental erosion and abrasion (ground sections).

FIG. 6. A lesion showing extensive erosion with a very smooth surface border. The demineralization extends to a maximum depth of $100\ \mu$ but varies down to almost undetectable depth in part of the lesion. In one region a thin surface zone appears slightly hyper-calcified as though some minerals may have become redeposited, presumably from saliva. $\times 50$.

FIG. 7. A case of erosion in which the demineralization is layered into one superficial zone of considerable demineralization and one deeper zone of lesser demineralization to a total subsurface depth of about $100\ \mu$. $\times 100$.

FIG. 8. A lesion with an irregular surface, possibly a combination of erosion with a more acute surface destruction. Unlike caries, the demineralization gradient is very evenly distributed along a superficial zone of less than $100\ \mu$. $\times 100$.

FIG. 9. A lesion grossly resembling erosion with a location and history suggestive of toothbrush abrasion. The surface of this lesion is sharply outlined with no demineralization gradient but, on the contrary, a suggestion of slight hyper-mineralization along the surface, possibly imparted by either a salivary or dentifrice agent.

FIG. 10. A lesion produced experimentally by a tooth-brushing machine (200 strokes/min, 150 g brush pressure, 33 per cent slurry of anhydrous dicalcium phosphate abrasive, total of 5000 strokes) shows a very similar situation to that of Fig. 9 with a sharply outlined surface and no evidence of subsurface drainage of minerals. (This tooth-brushing machine lesion was produced through the courtesy of the Procter and Gamble Company in the Miami Valley Laboratories, Cincinnati, Ohio).

FIGS. 11-14. Resorption of bone, dentine and enamel.

FIG. 11. Resorption along the alveolar bone surface of human jaw (ground section). Along the lacunar resorption cavities, there appears to be no distinct demineralization gradient; one of the Howship's lacunae at the extreme left involves, in a semi-circulated pattern, bone layers of unequal microradiographic density. Cementum, separated from the bone by the radiolucent periodontal membrane, is in this specimen remarkably intact. $\times 100$.

FIG. 12. Howship's lacunae along the dentine and enamel surface of a shed deciduous molar (ground section). The surface appears to be without any demineralization gradient. D—dentine; DEJ—dentino-enamel junction; E—enamel. $\times 250$.

FIG. 13. Decalcified section of enamel from a human deciduous tooth, which did not become shed until resorption had proceeded into the enamel on one side of the tooth. The enamel matrix shows Howship's lacunae with several giant cells (detached from the vascular part of granulation tissue normally underlying resorbing teeth). The enamel prism sheets run horizontally, and a pronounced region of the prism sheets, corresponding to the neonatal line of the enamel, runs in vertical direction, separating prenatal and post-natal (right) enamel. $\times 250$.

FIG. 14. Resorption of the alveolar bone of a rhesus monkey (decalcified section). The giant cells, situated in Howship's lacunae, are seen in near proximity to adjacent blood-vessels, and one of these cells (upper right) appears as though situated within a blood-vessel. $\times 500$.

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MICRORADIOGRAPHIC OBSERVATIONS ON DEMINERALIZATION GRADIENTS

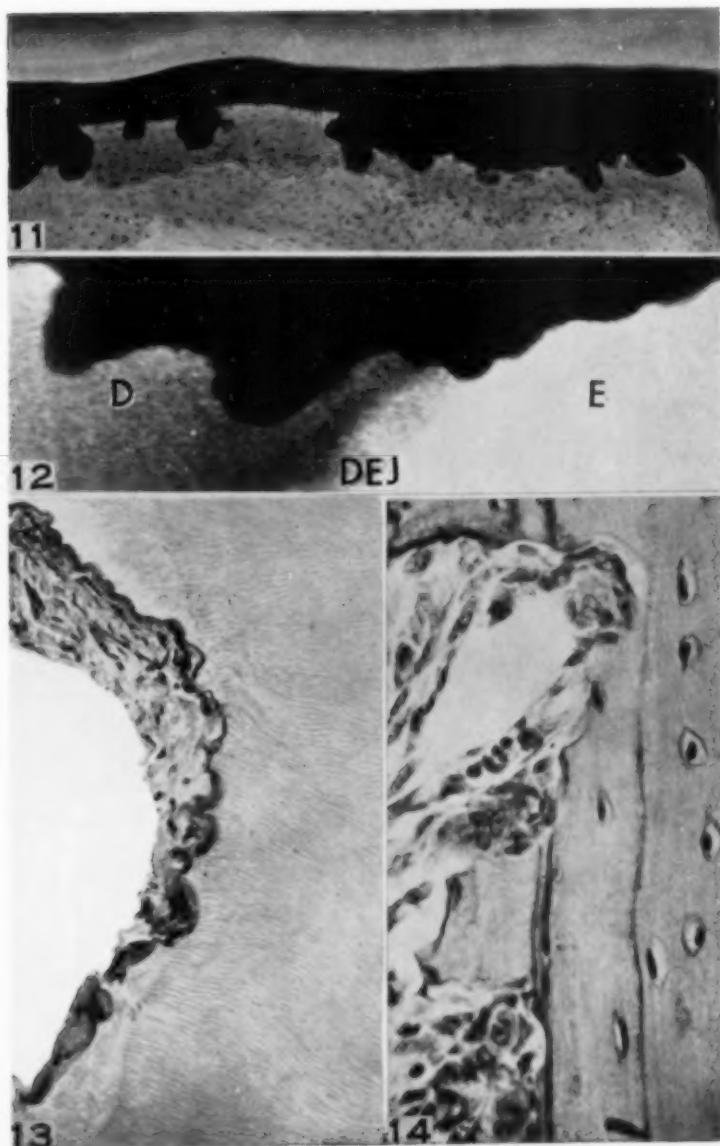
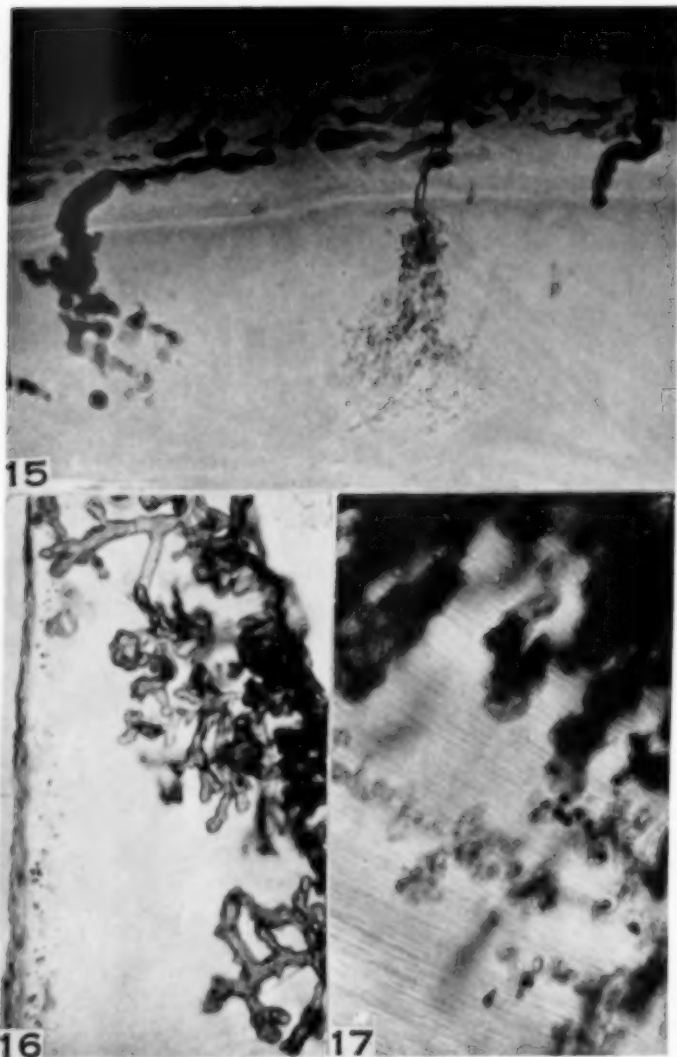


PLATE 3

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FIGS. 15, 16 AND 17. Post-mortem destruction of dentine and cementum (ground sections).

FIG. 15. Microradiograph showing post-mortem canals in an exhumed tooth (Middle Age Norway). There appears to be complete disintegration of the tooth substance along the excavating canals without any surface gradient in demineralization. $\times 90$.

FIGS. 16 AND 17. Post-mortem canals examined in transmitted light show the destructive pattern of the canals. Note that the destruction runs crosswise through the matrix without any relationship to the dentinal tubules. $\times 90$.

THE EFFECT OF INSULIN AND EPINEPHRINE ON RAT PAROTID GLYCOGEN*

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Abstract—The glycogen content of rat parotid gland has been determined. It averaged 83 ± 3 mg per cent in untreated, fasted animals. As the blood glucose is elevated glycogen is deposited in the gland, reaching a maximum when the blood glucose is about 500 or 600 mg per cent. Insulin significantly increases parotid glycogen at all blood sugar levels. Epinephrine lowers it at high blood sugar concentrations.

We reported previously (LANGLEY, GUNTORPE and BEALL, 1958) that there is normally no glucose in the parotid saliva of dogs. Glucose does appear in the saliva, however, when the blood sugar is raised above about 500 mg per cent. We further found that if insulin is given along with the infusion of glucose, the threshold is strikingly elevated. HEBB and STAVRAKY (1937) showed that the administration of epinephrine results in the appearance of glucose in the saliva even when the blood glucose is very low. They concluded that epinephrine increases the permeability of the salivary glands. On the other hand, the possibility exists that epinephrine may exert this influence on salivary glucose secretion by its effect on carbohydrate metabolism. As a step in the evaluation of this possibility we studied the effect of insulin and epinephrine on parotid glycogen.

METHODS

Male rats, weighing about 200 g, of the Long-Evans strain, maintained on Purina Laboratory Chow, were used. To elevate blood sugar, glucose was given subcutaneously 90 min before the parotid glands were removed. Insulin was administered subcutaneously in two doses, 32 units 45 min and 32 units 15 min before sacrifice. Epinephrine (1:1000) was given subcutaneously in two doses, 0.2 ml 90 min and 0.2 ml 45 min before sacrifice. The parotid glands were carefully dissected, then weighed on a microtorsion balance. Glycogen was analysed by the method of HANSON, SCHWARTZ and BARKER (1955), blood glucose by the anthrone procedure.

RESULTS

Fig. 1 discloses that in the normal fasted rat the parotid glycogen averages 83 ± 3 mg per cent (s.e.). As the blood glucose is elevated glycogen is deposited in the gland reaching a maximum when the blood glucose is about 500 or 600 mg per cent. A total of 26 rats were analysed in this untreated series.

* Supported by grant from National Institutes of Health, USPH-D-329 (C2).

The administration of insulin significantly alters the parotid glycogen at all blood sugar levels. It may be seen in Fig. 1 that fasted rats given insulin have more parotid glycogen than do the normals. If the parotid glycogen in the two groups is compared, this difference is found to be significant (Table 1).

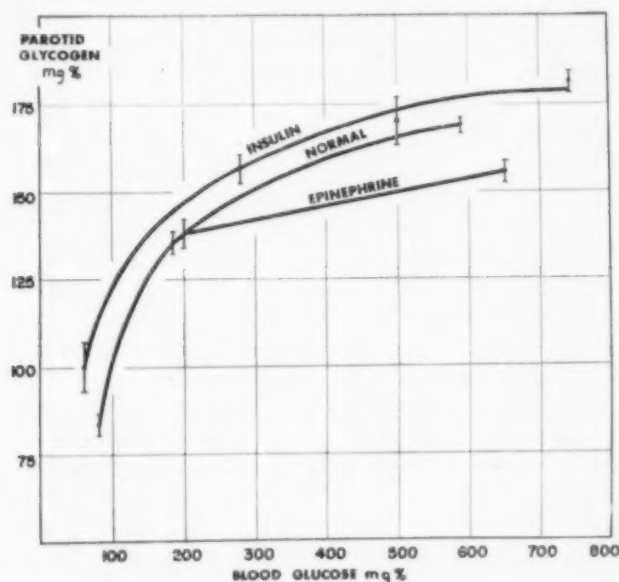


FIG. 1. The effect of insulin and epinephrine on rat parotid glycogen at different concentrations of blood glucose.

TABLE 1. THE EFFECT OF INSULIN AND EPINEPHRINE ON RAT PAROTID GLYCOGEN

Treatment	No. of animals	Blood glucose (mg %)	Parotid glycogen (mg %)	P
Untreated	6	587	169 \pm 2*	
Epinephrine	6	653	155 \pm 3	<0.02†
Insulin	5	742	181 \pm 3	<0.05†

* Standard error of the mean.

† Compared with the untreated average.

In contrast with the untreated and the insulin groups is the series in which epinephrine was given. Table 1 shows that the parotid glycogen in these animals is significantly lower than it is in the other groups.

DISCUSSION

A search of the literature disclosed surprisingly few reports of salivary-gland glycogen. BERGONZI and BOLCATO (1931) found no decrease in glycogen on stimulation of the gland. In contradistinction, NORTHUP (1935) reported that stimulation of the submaxillary glands decreased the glycogen content on the average of about 40 per cent. No data on parotid glycogen values have been found. NORTHUP reported that the dog submaxillary glands contained on the average 300 mg per cent glycogen before stimulation and about 180 mg per cent afterwards. HIMWICH and ADAMS (1930) found values closer to 100 mg per cent. These figures are to be compared with the levels we found in rat parotid gland. It is seen that the averages are not too dissimilar.

The deposition of glycogen in the parotid gland under the influence of insulin is not unexpected as this is a characteristic of the hormone, at least in skeletal muscle, if not in the heart (RUSSELL and BLOOM, 1956). Our results are also consistent with the finding that epinephrine produces a fall in the glycogen content of the gastrocnemius and diaphragm muscles (BLOOM and RUSSELL, 1955).

The question as to whether or not the parotid glycogen level plays any role in the secretion of glucose in the saliva remains unanswered. Both insulin and epinephrine have been shown to alter significantly the salivary glucose threshold; insulin raises it and epinephrine lowers it. It is found that under the influence of insulin the parotid glands accumulate more glycogen than do those of untreated rats, whereas epinephrine suppresses this accumulation at a blood glucose concentration of about 650 mg per cent. The findings for insulin are consistent with, but do not prove, the hypothesis that glucose which enters the cells of the salivary glands is converted to glycogen and thus is not available for secretion in the saliva until glycogen ceases to be formed. The findings for epinephrine at a blood glucose concentration of about 650 mg per cent support the hypothesis. But at a blood glucose concentration of 200 mg per cent the dose of epinephrine used in this study did not affect glycogen accumulation in rat parotid glands.

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A NEW LABORATORY TEST FOR ACID PRODUCTION IN SALIVA-CARBOHYDRATE MIXTURES AND ITS COMPARISON WITH THE LACTOBACILLUS COUNT AND THE SNYDER TEST*

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Abstract—A new laboratory technique is described for the determination of acid formation in saliva-carbohydrate mixtures. The results obtained with this method are compared with the lactobacillus count and the Snyder test. Adaptation of the salivary flora to sucrose in the diet can be detected by the method. The hydroxyapatite-agar plate may be useful in the assessment of diet and dietary changes in relation to dental caries.

SEVERAL methods have been used to study acid formation from carbohydrates in saliva and in the dental plaque. The simplest procedure is to incubate saliva with a suitable carbohydrate and, after a given period, to determine the pH. The difference between the original pH and that after incubation is a measure of the acid formed in excess of the buffering power of the saliva. If a preliminary titration is carried out so that a pH-acid curve is obtained, then the change in pH can be expressed in terms of equivalents of acid. Another way to measure acid produced in a given period is to titrate the acid with alkali, until the original pH value is reached or a predetermined pH is attained, e.g. pH 7.0. Acid formation may be linked with enamel dissolution, as in the Fosdick test (FOSDICK, HANSEN and EPPLE, 1937) in which a known weight of powdered enamel is incubated at 37°C for 4 hr with a glucose-saliva mixture. The dissolved calcium is estimated after precipitation as oxalate by the permanganate titration technique. *In vivo* the acid formed in the dental plaque may be measured by pH changes, using glass or antimony micro-electrodes (STEPHAN, 1944; STRALFORS, 1951; CHARLTON, 1956; KLEINBERG, 1958). Because the buffering capacity of the plaque and of the adsorbed salivary buffers is not readily determined *in vivo*, the plaque-pH change cannot be expressed in terms of equivalents of acid. Manometric methods (UMBREIT, BURRIS and STAUFFER, 1946) may also be employed to measure acid formation in saliva: the basis of this technique is that each molecule of acid formed liberates an equivalent amount of carbon dioxide which can be measured by manometers. Such a method is very sensitive and measures initial rates of glycolysis. Also this type of experiment is very adaptable to use in studies of

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inhibitors of acid formation and to elucidating particular aspects of the mechanisms of action of such inhibitors.

JOHNSTON (1952) used an agar-plate technique in his studies of the role of acids in solubilization of calcium phosphates. In this method the phosphates were wet-milled and incorporated into the agar. SPERBER (1957) studied the solubilization of phosphates by soil micro-organisms and employed an agar-plate technique which consisted of precipitated hydroxyapatite in the nutrient agar. This precipitation procedure of SPERBER was adapted by us to the preparation of homogeneous hydroxyapatite-agar plates for use in studies of acid formation by saliva and of certain aspects of hydroxyapatite solubility (LILIENTHAL and REID, 1958). This procedure has some advantages over those in which solid calcium phosphate (a hydroxyapatite) is incorporated into the medium. DAVIES, SLACK and TILDEN (1948) used such a medium for isolation of acidogenic organisms from saliva. The methods employed are described in this report and the results are compared with those of lactobacillus counts and of the Snyder test.

METHODS

A. *Preparation of hydroxyapatite-agar plates*

Add to each 100 ml of melted nutrient agar at 50°C 2.0 ml of 1 M calcium chloride and 2.0 ml of 0.6 M dipotassium hydrogen phosphate. Stir well and distribute 12 ml to each petri dish (4 in. in diameter). The petri dishes should be as uniform in size as possible and have flat bases. Care must be taken to eliminate bubbles by appropriate swirling of the medium in the petri dish. Place glass cylinders on the surface of the solidified agar: a preliminary heating ensures a seal with the agar. (The cylinders used were 7 mm external diameter and 5 mm internal diameter). It is recommended that the plates be prepared on the day they are to be used as it has been found that repeated melting of the hydroxyapatite-agar medium to prepare plates produces an unsatisfactory medium.

The calcium and phosphate added to the nutrient agar are in the same molar ratio as that in which calcium and phosphorus exist in hydroxyapatite, i.e. 1.66. By trial and error, 2.0 ml of each solution per 100 ml were found to produce a suitable amount of precipitate, and a fourfold variation of the amount of added calcium and phosphorus did not appreciably alter the size of the zones of solution, when a given saliva-glucose mixture was added to the glass cylinders.

B. *Determination of acid formation by saliva*

A 5 min sample of paraffin-stimulated saliva was collected from each of 225 subjects among several groups of individuals. For each individual test 0.03 ml of saliva was pipetted into a glass cylinder followed by 0.03 ml of 5% glucose or 10% sucrose. Such concentrations were found to give maximum (or near maximum) acid formation from saliva (BLANCH, LILIENTHAL and REID, 1958). The plates were incubated for 18 hr at 37°C, after which time the fluid had diffused from the glass cylinders into the agar. The size of each clear zone of dissolved hydroxyapatite is proportional to the amount of acid formed and, because in the experiments the depth of the agar was

kept constant, the area of the clear zone was a measure of the acid formed. The results were expressed in terms of the square of the radius (r^2) of the clear zone which is proportional to the area of this zone, viz. area of zone $= \pi r^2 \propto r^2$.

C. *Procedure for lactobacillus counts and Snyder tests*

The numbers of *Lactobacillus* spp. were counted by the pour-plate technique using 0.1 ml of diluted saliva on the selective medium described by ROGOSA *et al.* (1953). The Snyder test was carried out by inoculating brom-cresol-green glucose agar slabs with 0.1 ml undiluted saliva. "Difco" Snyder medium was employed and the colour changes were recorded at 24 and 48 hr during incubation at 37°C.

RESULTS

A. *Comparison of the results obtained by the lactobacillus count and the Snyder test*

The Snyder test measures the acidogenic power of saliva at pH 5.0: only those bacteria in saliva which can grow and ferment glucose at this pH are important in this test. The genus *Lactobacillus* represents the main group of oral bacteria which possess the dual property of aciduricity and acidogenicity. However there are also some species of *Streptococcus* and *Staphylococcus* which possess similar properties. Hence it would be expected that the lactobacillus count in most instances would be comparable with the Snyder test, the exceptions being caused by the presence of aciduric species of other bacteria, mainly *Streptococcus*. Furthermore, the use of whole saliva in this test is an attempt to examine the combined effects of the oral flora, including the stimulating effect of one organism upon another (synergism) and antagonism between organisms. Thus, for example, if two or more species of bacteria together produce a greater quantity of acid than the sum of the quantities which each could produce alone, then this phenomenon (synergism) might be detected in a test in which the organisms can grow together and ferment the carbohydrate. Conversely, antagonism between species should also be shown by a reduction in acid formation. However, neither synergism nor antagonism are detected as such by this method but their total effects are included in the result obtained.

Good agreement is apparent between low lactobacillus counts (i.e. less than 1000 organisms/ml) and a "negative" score in the Snyder test (Table 1). Likewise there is a good agreement between high lactobacillus counts (more than 10,000 organisms/ml) and the +++ and ++++ scores of the Snyder test. Furthermore, lactobacillus counts between 1000 and 10,000/ml are distributed over the range of Snyder scores in the proportions which might be expected. Exceptions are found in the following groups:

Lactobacillus count	Snyder test	No. of tests
(a) Less than 1000 organisms/ml	{ ++ +++	9 10
(b) 1000 to 10,000 organisms/ml	-	5
(c) More than 10,000 organisms/ml	+	5

Therefore twenty-nine (12 per cent) of the 236 comparisons did not show agreement and this disagreement was probably the result of acidogenic activity of bacteria other than the *Lactobacillus* in (a) and either antagonistic effects of other species or weak acidogenic activity of the *Lactobacillus* in (b) and (c).

TABLE 1. COMPARISON OF THE RESULTS OBTAINED BY THE LACTOBACILLUS COUNT AND BY THE SNYDER TEST

Lactobacillus count	Snyder test*				
Organisms per millilitre	—	+	++	+++	++++
< 1000	40	8	9	10	
1000–10,000	5	3	8	25	1
> 10,000		5	6	75	41

* Scoring method with Snyder medium:

24 hr	48 hr	Score
Green	Green	—
Green	Green-yellow	+
Green	Yellow-green	++
Green	Yellow	+++
Yellow	Yellow	++++

B. *Comparison of the hydroxyapatite-agar plate method, the lactobacillus count and the Snyder test*

The results which are set out in Table 2 show no obvious relationship between the hydroxyapatite-agar plate method and the Snyder test nor between the plate method and the lactobacillus count. Table 2 is first divided into three parts according to the magnitude of the lactobacillus count and in each a comparison is then made between the amount of acid formed in the plate test and the Snyder test. At each level of Snyder score or lactobacillus count, the greatest number of tests showed acid production equivalent to r^8 of between 30 and 40 mm².

C. *Comparison of acid formation from glucose and sucrose with the hydroxyapatite-agar plate method*

When the amounts of acid formed from 5% sucrose are compared with the corresponding amounts from 2.5% glucose (i.e. final sugar concentrations in the test) it is found that in the majority of tests more acid is formed from sucrose than from glucose. Only in a few instances were the amounts of acid from both sugars equal; with a few such subjects a change in their dietary pattern to one containing sucrose in large amounts resulted in acid values from sucrose larger than those from glucose.

When the excess sucrose was withdrawn the values returned to their original levels, i.e. glucose and sucrose values approximately equal. This phenomenon appears to be an indication of adaptation of the oral flora (or a section of it) to the utilization of sucrose either by increased bacterial invertase formation or by direct utilization such as in levansucrase action by *Strep. salivarius*.

TABLE 2. COMPARISON OF RESULTS OBTAINED BY PLATE TEST, LACTOBACILLUS COUNT AND SNYDER TEST

Frequency distribution				
A. Lactobacillus count <1000/ml				Snyder test
1	3	5	2	++++
	1	6	2	+++
	2	5		++
	9	24	2	+
				—
60-50	50-40	40-30	30-20	
Plate test (r^2 : mm ²)				
B. Lactobacillus count 1000-10,000/ml				Snyder test
		1		++++
	4	13	6	+++
		7	1	++
		1	2	+
	2	3		—
60	50	40	30	20
Plate test (r^2 : mm ²)				
C. Lactobacillus count <10,000/ml				Snyder test
2	6	26		++++
1	6	57	3	+++
1	1	2	2	++
		4		+
				—
60	50	40	30	20
Plate test (r^2 : mm ²)				

The frequency distribution of the difference between the amounts of acid produced from sucrose and glucose, i.e. $R_s^2(\text{sucrose}) - R_g^2(\text{glucose})$, was determined for each of the three groups of lactobacillus counts (Fig. 1). At lactobacillus counts of less than

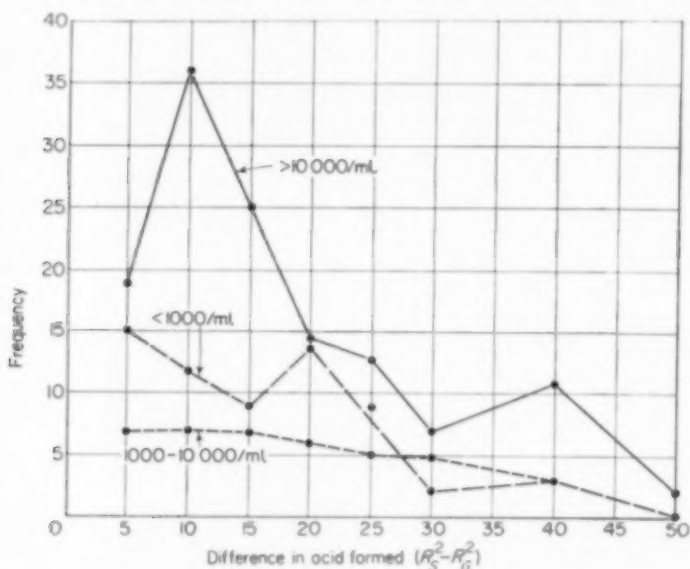


FIG. 1. Frequency distribution of differences in acid formed from sucrose and glucose by saliva at different lactobacillus counts.

- Lactobacillus count, 10,000 organisms/ml saliva.
- Lactobacillus count, 1,000-10,000 organisms/ml saliva.
- Lactobacillus count, 1,000 organisms/ml saliva.

R_s^2 = (radius)² of clear zone with sucrose as substrate.

R_g^2 = (radius)² of clear zone with glucose as substrate.

1000 organisms/ml there was an even distribution of $R_s^2 - R_g^2$ and a somewhat similar distribution was found in the 1000-10,000 organisms/ml group. However, when the magnitude of the lactobacillus count was greater than 10,000 organisms/ml there was in this group a greater frequency of the smaller differences in acid formation between sucrose and glucose, suggesting that high lactobacillus counts are not associated invariably with the formation of greater amounts of acid from sucrose than from glucose.

DISCUSSION

There is evidence which shows that the presence of *Lactobacillus* spp. in the saliva of an individual does not necessarily indicate susceptibility to dental caries (GOLDSWORTHY and SPIES, 1958). In fact, such susceptibility cannot always be assessed simply by either the lactobacillus count or the Snyder test or any of the established laboratory procedures which assess the acid-forming potential of the oral flora. In

communities enjoying western-style civilization and dietary habits, good agreement has been shown between the dental caries activity of the individual and the lactobacillus count or Snyder test. This relation appears to be based on a common factor shared by the process of dental caries on the one hand and the proliferation of *Lactobacillus* strains on the other. This common factor may be the frequency of ingestion of carbohydrate with the resultant accumulation around the teeth and in the saliva of readily soluble and fermentable carbohydrate.

The results of tests carried out with saliva-carbohydrate mixtures, rates of acid formation by oral bacteria and the method described above, all suggest that the oral flora has a sufficiently high potential for acid-formation provided the fermentable carbohydrate is present. It is suggested that the basic potential of the oral flora to form acid and thereby cause dental caries does not vary sufficiently from one subject to another to represent an important variable in the aetiology of dental caries. On the contrary, the retention of carbohydrate, the substrate for the bacteria, is indeed important and may be one of the major predisposing factors to the disease.

If this is correct then a method which will detect dietary influences would be useful. The lactobacillus count or the Snyder test or both appear to be satisfactory methods which reflect the dietary pattern. The hydroxyapatite-agar plate method for the assay of acid formation in saliva-carbohydrate mixtures gives results which are not directly related to either the lactobacillus count or the Snyder test. The observations from the plate method are however in agreement with those from methods such as the manometric determination of rates of acid formation, which method is also unrelated to the lactobacillus count (LILIENTHAL, 1956; LILIENTHAL and SPIES, 1957). Furthermore, the hydroxyapatite-agar plate method shows the ease with which the oral flora adapt to sucrose fermentation and this may be a useful technique for the detection of a dietary pattern with an excessive proportion of sucrose-containing foods.

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DIFFERENTIAL CALCIFICATION RATES IN THE HUMAN PRIMARY DENTITION*

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Abstract—The calcified portions of the crowns of the teeth of seventy-six human foetuses were removed after clearing and staining *in situ* with alizarin red S. Measurements were taken of the maximum mesio-distal diameter and the vertical depth of the enamel over the cuspal tips. The material investigated covered an age range of 13-18 weeks *in utero*. Statistical analysis indicated that the primary teeth do not calcify at the same rate either mesio-distally or vertically. The maxillary central incisor calcifies at a faster rate than do the other teeth in both dimensions. In all teeth studied, calcification proceeds faster mesio-distally than vertically. In terms of absolute chronology calcification mesio-distally shows a sigmoid type of growth curve not unlike that characteristic of postnatal skeletal growth.

IN view of the fact that no component of the body has been observed to exhibit a constant rate of growth, it is a matter of great interest to learn that the teeth, in their process of calcification, do not apparently reflect allometric growth. In the American literature, but not, significantly, in the European, it is commonly asserted that enamel is deposited in regular daily "rhythmic" intervals of $4\ \mu$ thickness, e.g. BRAUER *et al.* (1958), NOYES (1953) and ORBAN (1957). The explanation of the morphodifferentiation of the human dentition then becomes most difficult to piece together from the literature. The fact is that almost no serious attention has been devoted to the subject† and the statements made about such relevant subjects as the structure and function of the dentino-enamel junction, the sites of calcification, the initiation of calcification, the life-span of the ameloblasts and the rate of enamel deposition, when gathered together, do not form a coherent picture of crown development and differentiation.

The well-known assertion that in man enamel is deposited at the rate of $4\ \mu$ a day is the result of a gradual distortion of the results of the original work of SCHOUR and PONCHER (1937). These authors, working on the teeth of a single abnormal infant during the age period of 5-9 months, examined hundreds of stained, decalcified sections, and came to the following conclusions:

- (1) "The average rate of apposition of the enamel in the gingival portion of the deciduous lower second molar . . . was $3.92\ \mu/24\ \text{hr.}$

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† The only exception known to us is the recent work of MOSS and APPLEBAUM (1957) whose conclusions concerning allometric growth of the primary second molar and mandibular incisor are based upon extremely small samples.

- (2) "The average rate of apposition of the enamel of the permanent teeth present . . . was $2.71 \mu/24$ hr.
- (3) "Slight but consistent deviations from the average rate of apposition were observed; they appeared to correspond with the particular form and contour of a given tooth. These deviations are indicative of a definite gradient of growth for each type of tooth."

In addition the average rates of apposition of enamel for the lower permanent central incisor, canine, and first molar were given as 3.10, 2.80 and $2.70 \mu/24$ hr respectively. There was no statistical analysis of the data, nor any attempt to assess the experimental and sampling errors. Nevertheless, this work has remained the sole justification for all subsequent pronouncements about enamel apposition rates. It can be seen that in the original work the authors interpreted their data, rightly or wrongly, in quite a different manner from that currently found in the dental textbooks. SCHOUR and PONCHER definitely imply differential growth rates for the appositional process in the various teeth and in different parts of the same tooth, although in the absence of statistical verification they were justified only in making speculations. Apparently SCHOUR repudiated his earlier opinion about differential growth rates, for he states:

"The amount of growth is definitely set by the rate of work (averaging 4μ per day in man) and the functional life-span of the formative cells." (SCHOUR, 1957, p. 54).

Elsewhere SCHOUR is more definite, for example:

"Incremental caps of enamel one globule (4μ) in thickness are apposed daily over each growth centre, one above another. The functional life span of the ameloblasts limits the particular length of the enamel rod and thus determines the thickness of the enamel." (BRAUER *et al.*, 1958, pp. 55-56).

Here the thickness of the enamel is stated to be a function of the life-span of the ameloblasts, but a few pages later we read:

"Enamel and dentin formation proceeds regularly and rhythmically after its inception, and the time required for the completion of the crown depends on the length of the crown and the rate of tissue formation." (BRAUER *et al.*, 1958, p. 63).

Now it appears that the time of crown completion is a function of the length of the crown and the rate of tissue formation. It is obvious, however, that the crown length is the dependent variable. If the rate of tissue formation is constant, as was stated in the previous quotation, then it is an independent variable.

There are further difficulties of interpretation with regard to the life-span of the ameloblasts. The most definitive statement is by Bhaskar:

"Each ameloblast of the enamel organ functions for a given length of time, i.e. produces enamel rod substance, and then ceases to function. The functional life span of ameloblasts varies; it is longest at cusp tips and shortest at the cervical part of the crown. Correspondingly, the thickness of enamel matrix and therefore of enamel varies in different teeth and different parts of the same tooth." (COHEN, 1957, p. 112).

We are unaware of the evidence for the differential life-span of ameloblasts, but let us assume this to be true. We then are confronted with the following oft-asserted points:

- (1) The rate of enamel apposition is constant, at $4 \mu/24 \text{ hr}$.
- (2) The dentino-enamel junction is the blueprint pattern for the tooth, predetermining its characteristic form and size. (ORBAN, 1957, p. 53).
- (3) There is differential longevity of the ameloblasts specific to different teeth and to different parts of the same tooth.

If we concede the correctness of these statements then it is difficult to understand, commencing with the advanced bell stage of morphodifferentiation of the future crown, how the typically human size and pattern of the individual tooth crowns are achieved. The soft-tissue crowns prior to calcification are, in fact, much more similar to those of the primitive tarsiers (Fig. 1) than are the eventual hard-tissue



FIG. 1. Comparison of first permanent molars of a human (soft-tissue crown) and a fossil primate (calcified crown).

(A) buccal view of the right mandibular first molar of *Tetoniuss homunculus*, a Lower Eocene tarsier found in Wyoming (after GREGORY, 1922, Fig. 119).

(B) buccal view of a right mandibular first permanent molar before the onset of calcification (taken from a full-term human stillborn).

crowns (KRAUS, 1959b). This being so, if calcification continues longest at the cusp tip and proceeds at the same rate as elsewhere on the crown, then the human molar, for example, would be strikingly similar to that of such forms as the Jurassic *Amphitherium* or the Eocene *Tarsiodea* (GREGORY, 1951).

The morphology of the dentino-enamel junction and the nature, pattern and rates of enamel deposition thereon must be re-examined. A clear understanding of these aspects of tooth development should add immeasurably both to our knowledge

of the evolution of the dentition and to our comprehension of the role of genes in morphodifferentiation.

The purpose of this paper is to examine the progress of calcification during pre-natal development in its gross aspects. Indirectly an attempt will be made to determine whether this process, too, is characterized by differential growth rates.

METHOD AND MATERIAL

A total of 76 foetuses, ranging in crown-rump length from 88 to 163 mm, were cleared and stained with alizarin red S. The teeth were then removed from the crypts and examined under low magnification (less than $\times 80$). By means of a micrometer disc inserted into a $\times 15$ eyepiece on the Cycloptic Stereoscopic Microscope, measurements were taken of the maximum mesio-distal diameter of the calcified portion of the tooth. In addition, the maximum vertical thickness of the enamel measured from the tip of the dentino-enamel cusp, was recorded. Repeated measurements on a randomly selected sample by different observers confirmed the error to be less than 0.05 mm. The two types of measurement are illustrated in Fig. 2. No attempt was

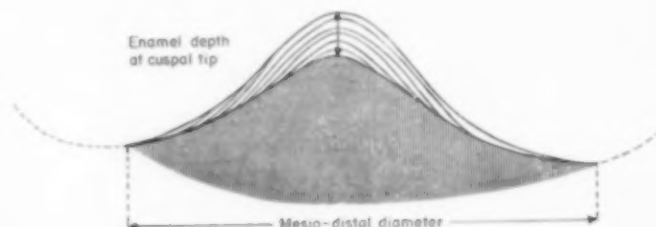


FIG. 2. A diagrammatic representation of the measurements made on primary teeth. The shaded portions indicate the stained (calcified) areas of the tooth.

made to determine whether or not the staining of the calcified areas of the teeth was affected by the fixative agent, an objection raised for the skeletal portions by O'RAHILLY and MEYER (1956). The staining technique was preferred to roentgenography for reasons stated by NOBACK (1954) and KRAUS (1959a).

The following primary teeth were used in this study: maxillary and mandibular central incisors, maxillary lateral incisor and maxillary first molar. For the age period studied these are the only teeth (with the exception of the lower lateral incisor) which are consistently in some stage of calcification. On the maxillary first molars only the mesio-buccal cusp is undergoing calcification during this period. On the canines only in the larger foetuses of the sample had initial calcification occurred. The sequence of initial calcification of the primary teeth has been discussed by KRAUS (1959a).

The statistical methods employed include the analysis of variance on multiple regressions, the comparison of slopes, and the test for curvilinearity of regression. The basic data are presented in Table 1.

TABLE I. CROWN-RUMP LENGTH, MESIO-DISTAL DIAMETER AND VERTICAL ENAMEL DEPTH ON 76 HUMAN FOETUSES (in mm)
(nm. = not measured)

Foetus No.	C-R length	Mesio-distal diameter					Vertical enamel depth	
		Max. l-1	Max. l-2	Max. c	Max. m-1	Mand. l-1	Max. l-1	Max. m-1
41	88	1.45	0.30	0	0.32	1.09	0.084	0.042
85	94	1.41	0.50	0	0.46	0	0.063	0.042
X 9	95	nm.	nm.	nm.	nm.	nm.	0.084	0.000
X 26	95	nm.	nm.	nm.	nm.	nm.	0.105	0.052
X 30	97	nm.	nm.	nm.	nm.	nm.	0.047	0.005
X 47	91	nm.	nm.	nm.	nm.	nm.	0.257	0.031
105	110	3.15	1.92	0.44	1.95	2.11	0.084	0.063
132	111	3.24	1.67	0.61	2.00	2.54	0.252	0.231
X 12	110	nm.	nm.	nm.	nm.	nm.	0.021	0.000
X 13	107	nm.	nm.	nm.	nm.	nm.	0.084	0.063
X 15	104	nm.	nm.	nm.	nm.	nm.	0.052	0.021
X 20	105	nm.	nm.	nm.	nm.	nm.	0.063	0.042
X 23	103	nm.	nm.	nm.	nm.	nm.	0.031	0.031
X 24	101	nm.	nm.	nm.	nm.	nm.	0.073	0.000
X 28	100	nm.	nm.	nm.	nm.	nm.	0.084	0.000
X 29	100	nm.	nm.	nm.	nm.	nm.	0.115	0.084
X 79	103	nm.	nm.	nm.	nm.	nm.	0.168	0.094
X 80	102	nm.	nm.	nm.	nm.	nm.	0.105	0.031
52	114	2.90	1.16	0.35	1.05	2.58	0.252	0.105
54	115	0.89	0.27	0	0.35	0.60	0.063	0.021
125	114	2.30	0.85	0	0.62	1.39	0.126	0.084
X 11	116	nm.	nm.	nm.	nm.	nm.	0.126	0.042
X 22	119	nm.	nm.	nm.	nm.	nm.	0.094	0.010
X 88	121	nm.	nm.	nm.	nm.	nm.	0.168	0.105
62	133	2.35	1.03	0	0.69	1.91	0.231	0.105
59	128	2.33	1.16	0	0.64	2.00	0.147	0.084
60	127	2.00	0.81	0.49	0.71	1.87	0.147	0.105
78	125	3.49	2.05	0.65	1.70	2.51	0.315	nm.
61	128	2.49	1.49	0	1.14	2.10	0.252	0.147
64	135	1.74	0.50	0	0.53	1.01	0.126	0.126
X 36	134	nm.	nm.	nm.	nm.	nm.	0.168	0.105
X 37	139	nm.	nm.	nm.	nm.	nm.	0.210	0.147
X 40	140	nm.	nm.	nm.	nm.	nm.	0.105	0.073
X 42	127	nm.	nm.	nm.	nm.	nm.	0.105	0.042
X 43	140	nm.	nm.	nm.	nm.	nm.	0.105	0.105
127	140	2.12	1.29	0	0.59	2.06	0.189	0.105
151	133	2.13	1.26	0	0.84	1.45	0.189	0.126
63	133	3.61	2.52	0.48	1.97	2.73	0.315	0.189
102	149	4.10	2.65	0.74	2.35	2.65	0.189	0.126
66	144	2.97	1.59	0.46	0.84	1.95	0.378	0.273
69	153	4.20	3.11	1.43	3.40	3.02	0.378	0.315
75	145	3.78	2.46	0.67	2.35	2.70	0.378	0.315
118	152	1.99	0.69	0	0.75	0.47	0.147	0.105
149	143	2.30	0.78	0	0.80	1.70	0.189	0.105
65	142	3.36	1.80	0.34	1.35	2.21	0.315	0.168
121	142	3.02	0.88	0	0.80	1.93	0.189	0.084
131	142	2.40	1.60	0	1.03	2.37	0.315	0.231
68	151	2.35	1.97	0	1.73	2.43	0.231	0.168
X 32	146	nm.	nm.	nm.	nm.	nm.	0.084	0.063
X 35	153	nm.	nm.	nm.	nm.	nm.	0.189	0.126
X 38	147	nm.	nm.	nm.	nm.	nm.	0.378	0.231
X 39	147	nm.	nm.	nm.	nm.	nm.	0.189	0.126
X 46	143	nm.	nm.	nm.	nm.	nm.	0.231	0.210
X 87	148	nm.	nm.	nm.	nm.	nm.	0.115	0.105
X 96	145	nm.	nm.	nm.	nm.	nm.	0.210	0.126
70	154	3.70	1.99	0.38	1.97	2.35	0.231	0.168
101	154	3.49	2.19	0	1.82	2.56	0.294	0.189
74	163	4.28	2.86	1.26	3.49	2.90	0.315	0.252
72	158	3.40	1.28	0	0.73	1.20	0.294	0.189
144	157	2.43	1.28	0	0.63	1.53	0.168	0.084
136	160	1.91	0.80	0	1.98	2.38	0.357	0.210
103	155	3.99	2.70	0.67	1.98	2.38	0.231	0.147
X 33	154	nm.	nm.	nm.	nm.	nm.	0.042	0.084
X 41	154	nm.	nm.	nm.	nm.	nm.	0.189	0.147
X 44	155	nm.	nm.	nm.	nm.	nm.	0.210	0.168
X 45	158	nm.	nm.	nm.	nm.	nm.	0.273	0.220
X 94	160	nm.	nm.	nm.	nm.	nm.	0.105	nm.
49	111	1.77	nm.	nm.	nm.	nm.	0.042	nm.
51	113	0.84	nm.	nm.	nm.	nm.	0.021	nm.
141	104	0.48	nm.	nm.	nm.	nm.	0.084	nm.
147	118	1.28	nm.	nm.	nm.	nm.	0.042	nm.
143	130	0.77	nm.	nm.	nm.	nm.	0.063	nm.
100	143	0.59	nm.	nm.	nm.	nm.	0.147	nm.
71	157	1.86	nm.	nm.	nm.	nm.	0.063	nm.
126	nm.	1.07	nm.	nm.	nm.	nm.	0.021	nm.
79	99	0.67	nm.	nm.	nm.	nm.		

RESULTS AND DISCUSSION

If it were possible to follow the calcification process in a single individual and to correlate the vertical increase of enamel deposition on the maxillary central incisor cuspal tip with that on the mesio-buccal cuspal tip of the first molar, then a plot of the resultant cross-products should result in a linear regression line with a slope equal to unity, that is, if the rates of apposition are the same and are constant. Since a serial study is manifestly impossible, a cross-sectional approach must be attempted. This means that each cross-product represents a different individual. Since the individual fetuses are of varying ages and there is variability with respect to the extent of calcification of the central incisor when initial calcification of the first molar begins, a question arises. Will a least squares line determined from such co-ordinates reflect the true relationship of the rates? In a large sample, for any one X value there should be several Y values if the sample is randomly chosen. Furthermore there would tend to be a balance between those individuals in whom the molar began calcification long after the incisor and those in whom the molar commenced to calcify immediately after the incisor. A least squares line, then, should describe the relationship with validity. An analysis of variance gives the following results:

Source of variation	D.F.	S.S.	M.S.	F
Linear regression	1	751.1460	751.1460	375
Deviations from regression	64	128.3247	2.005	1
Total	65	879.4707		

Linear regression is, therefore, a significant source of the total variance. The test for curvilinearity (SNEDECOR, 1955, 374 ff.) fails to demonstrate significant curvilinear regression:*

Source of variation	D.F.	S.S.	M.S.	F
Deviation from linear regression	64	128.58		
Deviation from curved regression	63	127.43	2.02	1.0
Curvilinearity of regression	1	1.15	1.15	

The ratio of increase in enamel depth of the molar cusp to that of the central incisor is therefore constant throughout the age period represented. The slope of the line is 0.7391. A " t " test of the difference between unity and 0.7391 gives a t value of 6.83, indicating a probability of less than 0.001. Therefore, enamel deposition on the

* Values used in this regression analysis are in units of the micrometer. Each unit = 0.021 mm. In subsequent analyses the values are in millimetres.

mesio-buccal cusp of the first molar proceeds at a slower rate than it does on the central incisor, the ratio (*circa* 3:4) between the two rates being constant. The prediction formula for the first molar is: $Y=0.7391X-0.015$, where the value of X is in millimetres.

We next test the regression of the first molar on the central incisor in terms of the maximum mesio-distal diameter of the calcified dentino-enamel junction. The prediction formula for the first molar is: $Y=0.656X-0.4324$. The slope is 0.656 and differs significantly from unity ($t=9.2$, $P<0.001$). As in the case of enamel depth over the cuspal tip, the mesio-distal diameter of the central incisor increases at a greater rate than does that of the mesio-buccal cusp of the first molar. The ratio (*circa* 66:100) between these two rates is also constant, no significant curvilinear regression being demonstrable.

The ratios (or slopes) of the rates of enamel apposition vertically and mesio-distally may now be compared by the method of analysis of variance of pooled regressions, as follows:

Source of variation	D.F.	S.S.	M.S.	F
Pooled regressions				
(1) Joint regressions	1	784.5460	784.5460	
(2) Differences between regressions	1	0.5141	0.5141	0.44
Pooled deviations from regression	116	134.0628	1.1557	1.00
Total	118	919.1229		

An F ratio of less than unity indicates there is no significant difference between the slopes. This means that increase both in enamel depth and in mesio-distal diameter of the first molar proceeds at a slower rate than it does on the central incisor. In addition, these rates, relative to those of the central incisor, are the same—approximately 70 per cent of the incisor rates $(0.66+0.74)/2$.

Although calcification proceeds at a faster rate on the central incisor, whether vertically or mesio-distally, when compared with the first molar, the rates of deposition on the same tooth are significantly different. An analysis of variance on the regression of enamel vertical depth on mesio-distal diameter for the central incisor shows a significant linear regression:

Source of variation	D.F.	S.S.	M.S.	F
Linear regression	1	0.34265	0.34265	105.1
Deviations from regression	40	0.13053	0.00326	1.0
Total	41	0.47318		

The slope is 0.08469 and differs significantly from zero ($t=10.26$, $P < 0.001$), and from unity ($t=110.9$, $P < 0.001$). There is no significant curvilinearity of regression:

Source of variation	D.F.	S.S.	M.S.	F
Deviation from linear regression	40	13.1266		
Deviation from curved regression	39	12.3124	0.3157	1.0
Curvilinearity of regression	1	0.8142	0.8142	2.58

An F value of 2.58 (1/39 D.F.) has a probability greater than 0.10. The rate of enamel deposition on the cuspal tip is therefore considerably less than the rate of calcification in the mesio-distal direction. However, the ratio between the two rates is constant, and the relationship between the two observed dimensions of calcification can be expressed by a simple linear equation.

Thus far we have compared tooth with tooth, and different dimensions within the same tooth. Time has been eliminated. Now we might interject the time factor, using crown-rump length as a crude indicator of age. Testing for significant linear regression of the mesio-distal diameter of the maxillary central incisor on crown-rump length, we obtain the following results:

Source of variation	D.F.	S.S.	M.S.	F
Linear regression	1	26.3137	26.3137	26.93
Deviations from regression	51	49.8373	0.9772	1.0
Total	52*	76.1510		

* Additional foetuses, not listed in Table 1, were utilized to increase the sample size beyond forty-two.

Testing further for curvilinearity, we find:

Source of variation	D.F.	S.S.	M.S.	F
Deviation from linear regression	51	49.8561		
Deviation from curved regression	50	23.1880	0.4638	1.0
Curvilinearity of regression	1	26.6681	26.6681	57.5

There is significant curvilinearity of regression of incisor diameter on crown-rump length. This means that the rate of calcification of the central incisor in its mesio-distal diameter is not constant but changes during the prenatal period under study. Equating crown-rump length to age in weeks (PATTEN, 1946, p. 154), and using mean mesio-distal diameters for each age, we find a remarkable similarity of sigmoid curves

for the various teeth (see Fig. 3), which recalls those of SCAMMON and CALKINS (1929) for the external dimensions of the foetus. There is marked acceleration at 15 and 16 weeks and a deceleration thereafter. The curves for the cuspid and second molar are not statistically significant, however, because of inadequacy of sampling (these two teeth are relatively late in initial calcification, KRAUS, 1959a).

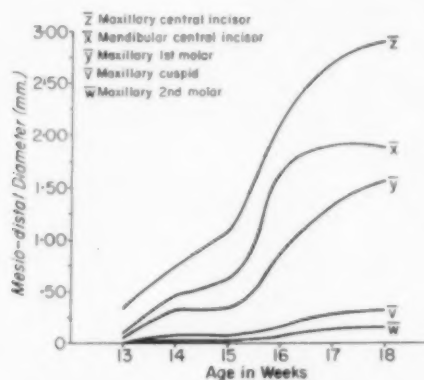


FIG. 3. Regressions of mesio-distal diameters of various primary teeth on prenatal age in weeks.

Recognizing that the various teeth in the primary dentition are not wholly independent of each other growthwise (BUTLER, 1939; OSBORNE, HOROWITZ and DE GEORGE, 1958), it would be of interest to determine the nature of the relationship in time between them. We may focus on that dimension which expresses the maximum mesio-distal extension of the calcified portion of the crown. Employing the multiple regression technique, it would be expected that a linear regression plane would best fit all the data if the rates of calcification in this dimension are the same. Letting the maxillary central incisor represent Y , the dependent variant, and the maxillary lateral incisor, maxillary first molar and mandibular central incisor be X_1 , X_2 and X_3 respectively, the basic measurements are set forth in Table 2. As expected, linear regression is demonstrated to be significant and we go on to test for significance of curvilinear regression:

Source of variation	D.F.	S.S.	M.S.	F
Deviation from linear regression	31	4.0694		
Deviations from curved regression	30	3.3841	0.1128	1.0
Curvilinearity of regression	1	0.6853	0.6853	6.07

The F ratio of 6.07 (1/30 D.F.) yields a P value of less than 0.03. There is, then, a significant curvilinear regression. This means that as calcification of the maxillary central incisor spreads mesio-distally, the corresponding diameters of the other teeth also increase but not at the same rates. In other words, given the dimension of one tooth, the others could be predicted with a stated confidence, by fitting an appropriate quadratic or cubic equation to the data.

The linear regression equation is:

$$Y = 0.9658 + 0.6918X_1 + 0.1187X_2 + 0.3017X_3.$$

TABLE 2. MAXIMUM MESIO-DISTAL CALCIFICATION OF FOUR PRIMARY TEETH (mm)
 $n=33^*$

X_1	X_2	X_3	Y	\hat{Y}	$(Y-\hat{Y})$
0.34	0.29	0.67	1.34	1.44	-0.10
0.30	0.32	1.09	1.45	1.54	-0.09
1.92	1.95	2.11	3.15	3.16	-0.01
1.67	2.00	2.54	3.24	3.12	+0.12
1.16	1.05	2.58	2.90	2.67	+0.23
0.27	0.35	0.60	0.89	1.37	-0.48
2.05	1.70	2.51	3.49	3.34	+0.15
0.85	0.62	1.39	2.30	2.05	+0.25
1.03	0.69	1.91	2.35	2.34	+0.01
1.16	0.64	2.00	2.33	2.45	-0.12
0.81	0.71	1.87	2.00	2.17	-0.17
1.49	1.14	2.10	2.49	2.77	-0.28
0.50	0.53	1.01	1.74	1.68	+0.06
2.65	2.35	2.65	4.10	3.88	+0.22
1.29	0.59	2.06	2.12	2.55	-0.43
1.26	0.84	1.45	2.13	2.38	-0.25
2.52	1.97	2.73	3.61	3.77	-0.16
1.59	0.84	1.95	2.97	2.75	+0.22
3.11	3.40	3.02	4.20	4.43	-0.23
2.46	2.35	2.70	3.78	3.76	+0.02
0.69	0.75	0.47	1.99	1.67	+0.32
0.78	0.80	1.70	2.30	2.11	+0.19
1.80	1.35	2.21	3.36	3.04	+0.32
0.88	0.80	1.93	3.02	2.25	+0.77
1.60	1.03	2.37	2.40	2.91	-0.51
1.97	1.73	2.43	2.35	3.27	-0.92
1.99	1.97	2.35	3.70	3.28	+0.42
2.19	1.82	2.56	3.49	3.47	+0.02
2.86	3.49	2.90	4.28	4.23	+0.05
1.46	2.17	2.90	3.40	3.11	+0.29
1.28	0.73	1.20	2.43	2.30	+0.13
0.80	0.63	1.53	1.91	2.06	-0.15
2.70	1.98	2.38	3.99	3.79	+0.20

* Although seventy-six fetuses were available, only thirty-three were measured with respect to the four teeth used in this analysis.

The predicted values of the maxillary central incisor are given in Table 1 under the column \hat{Y} , and the differences between observed and predicted under the column $(Y-\hat{Y})$. A "t" test of the coefficients of the X s gives the following results:

$$t_1 = 5.194 \quad (P < 0.01)$$

$$t_2 = 0.729 \quad (P > 0.40)$$

$$t_3 = 2.015 \quad (P \approx 0.05)$$

Since the coefficient for X_2 (maxillary first molar) is not significant it adds little, if anything, to the prediction equation. On the other hand, a curvilinear regression equation should give even better results, i.e. a better fit of predicted with observed results. Further tests of this nature with the permanent dentition might reveal whether or not the molar teeth can be grouped together, in terms of growth rates. If so, such groupings of the anterior and molar teeth would tend to substantiate the findings of OSBORNE *et al.* (1958) and DAHLBERG (1949) and would be in accord with the results of studies of prenatal growth factors operating on the various regions of the skeleton (KRAUS and CHOI, 1958).

In summary, this investigation was stimulated by the oft-repeated assertion throughout the dental literature that calcification of the teeth proceeds at a regular, rhythmic rate of 4 μ /24 hr. In view of the fact that this statement is the result of a gradual process of distortion of the original findings of SCHOUR and PONCHER in 1937, which in turn were statistically inadequate, and because this would constitute the one known exception to the biological phenomenon of differential growth of the organism and its parts, the present study was aimed at a gross analysis of the calcification process in the primary dentition.

Admittedly the findings of the present study are not conclusive and are not intended to set up parameters of growth rates in the primary teeth. The major purpose was to determine if gross observations of the calcification status of the various dental units support the thesis of a regular, rhythmic, constant rate of growth.

Statistical analysis of two sets of observations—the maximum calcified mesio-distal diameter and the vertical diameter of the calcified area immediately above the tip of the cusp of each tooth—was conducted on a total of 76 embryos ranging from 88–163 mm in crown-rump length. The teeth had been cleared and stained, *in situ*, with alizarin red S.

It was found that neither mesio-distally nor vertically do all the primary teeth calcify at the same rate. During the prenatal age period studied (13–18 weeks) the maxillary central incisor appears to calcify at a faster rate than do the other teeth in both dimensions. Furthermore, on any given tooth, calcification proceeds faster mesio-distally than vertically. The ratio of the rates of calcification in any one dimension for any two teeth is constant, as indicated by the fact that there is a significant linear but not curvilinear regression of one tooth upon the other.

When the mesio-distal diameters of any one tooth are plotted against time (age in weeks) a significant curvilinear regression results, not unlike the typical sigmoid growth curves of postnatal development. This indicates alternating acceleration and deceleration of the calcification process, a pattern which is characteristic of postnatal skeletal growth.

Analysis of variance on multiple regression of several teeth with respect to mesio-distal diameters indicates a significant curvilinearity of the regression plane. It is suggested that further study of this type on the permanent dentition might cast additional light upon the "field" concept of the dentition advanced by BUTLER and others. It may be possible that genetic factors exert a "regional" control over the differential calcification rates that apparently prevail in the primary dentition.

On the basis of the results obtained, it is perhaps reasonable to urge a re-examination and re-valuation, at the microhistological level, of the growth patterns exhibited by the teeth as they undergo calcification.

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GLYCOLYSIS INHIBITORS AMONG COMPOUNDS CONTAINING ALDEHYDES, KETONES, AND ORGANIC ACIDS*†

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Abstract—Nearly 800 compounds have been studied for ability to inhibit glycolysis of salivary sediment. These compounds possessed aldehyde, ketone and/or carboxyl groups as a part of the organic structure. The compounds were tested mainly at 1% concentration or half-saturation, in water or in 10% propylene glycol.

The most active aldehydes were formaldehyde, succinaldehyde, pyruvic aldehyde, mucochloric acid, 5-nitrosalicylaldehyde and *m*-hydroxybenzaldehyde. Inhibition can be attributed to the aldehyde group of the first three compounds. Nearly all active aldehydes possessed a high degree of specificity.

Among ketones there were nine organic structures which were capable of producing inhibition amounting to 50 per cent or more, but only for one substance, dioleoyl ketone, could inhibition be attributed definitely to the keto group.

Among others, such as pyruvic aldehyde, 2,3-butanedione-2-methoxime, Rose Bengal, 2-(dibutylamino)-ethylphenylketone and 2,4'-dihydroxybenzophenone, the inhibitory action was attributable to some group other than ketone. The three remaining inhibitory chemicals, verbenone, benzoquinone and 2,5-dimethylparaquinone possessed ketone as the sole functional group.

Among the eight organic acids showing inhibitory activity, there was a high degree of specificity. One group, bromacetate, mucochlorate and alphabromopropionate, was related to iodoacetate, but all were less inhibitory. Sodium N-lauroylsarcosinate was inhibitory, but few similar structures were available for comparison. The 3,5-di-iodo-salicylic acid probably owes its activity to the presence of the two halogens since loss of one of these produces an inactive structure, but 2-butyl-*x*-chlorophenoxyacetic has activity which is not possessed by closely related compounds. The two substances, 2-cyclopentene-1-valeric acid and cyclohexanebutyric acid are the only structures whose activity could apparently be attributed to the carboxyl group.

INTRODUCTION

THIS investigation is one aspect of a search for glycolysis inhibitors among a wide variety of compounds, totalling nearly 3300, which were screened in order to find those that might prove valuable as inhibitors of dental caries. This presentation is concerned with aldehydes, ketones and organic acids, the three types of organic compounds that can be considered as oxidation products of alcohols. The incidence of inhibitory action among these compounds is not high, but the fact that any

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inhibitory compounds are found among such simple organic structures is of academic interest. There are available for consideration 115 tests on aldehydes, 237 on ketones and 515 upon organic acids. Most of these compounds possessed other functional groups in addition to the structure of primary interest. In some cases inhibitory action could be attributed to the aldehyde, ketone or organic acid, and in other instances the effect is presumably due to some other grouping.

Previous studies have included surveys of activity among alcohols, phenols, ethers and esters (MANLY and HARGREAVES, 1957), amines (MANLY, 1954b), amides, ureas and urethanes (MANLY and HARGREAVES, 1956) and halogen derivatives (MANLY, DOLEMAN and HARGREAVES, 1956). Findings on aldehydes, ketones and organic acids have been presented orally (MANLY and HARGREAVES, 1955), but at that time structures containing some other elements, such as halogen or sulphur, were not considered because their effect on activity could not be predicted. Now that these structures have been analyzed, it is possible to broaden the scope of the analysis in order to include all structures containing a carbonyl group. Discussions will be confined to a few key examples of the three types of compounds.

METHODS

The procedure employed has been described elsewhere (MANLY, 1954a; HARGREAVES and MANLY, 1956). The principle of the test is to study rates of glycolysis in thin films prepared from centrifugates of whole, stimulated saliva obtained from human volunteers. Pooled centrifugate was coated upon a glass electrode and immersed in a buffered sugar solution until a steady state pH had been attained. The differential between the pH of the sediment and that of the solution was taken as an index of the control glycolytic activity of the centrifugate. Chemicals for test were generally tested either at 1% concentration or at half-saturation, whichever was less. Water was the preferred solvent, and solutions were first prepared at 2% concentration and titrated to pH 7-8. Two grams of compounds known to be, or suspected of being, slightly soluble in water were tried for solubility in 20 g of propylene glycol with stirring. The mixture was then diluted 4:1 with water to make a 20% propylene glycol solution. If not all of the chemical was dissolved after stirring, warming and neutralizing, the solution was filtered and marked "saturated". Next, equal volumes of the 2% or saturated solution were mixed with a solution of 0.02 molar H_2CO_3 - NaHCO_3 buffer containing 10% supernatant from saliva. This dilution produced buffered, supernatant-containing solution with 10% propylene glycol and either 1% chemical or "half-saturated" (S/2).

The solution so prepared was placed in contact with the centrifugate for a period of 20 min. Then the test solution was removed and replaced by control buffer glucose solution and attainment of a new steady state was permitted.

The ratio of second and first control pH differentials (times 100) was employed as the index of recovery of glycolytic activity. For example, if the buffer had a pH of 7.5 and the steady state pH of sediment was 5.5, the control differential would be 2.0 units. If we supposed the treatment did not change the buffer pH, but raised the sediment pH by one unit, then the recovery would equal $100 \times 1.0/2.0$ or 50 per

TABLE 1

Concentration of chemicals during test are indicated by asterisks:

No asterisks indicates test at half-saturation.

One asterisk (*) indicates test at 1.0%.

Two asterisks (**) indicate test at 0.1%, 0.01% or one-tenth saturation.

Part A—Aldehydes (RCHO)

(1) *Recovery of 0-39 per cent—high activity*

(a) Tested in water solution

Chloroacetaldehyde**
Mucochloric acid

Pyruvic aldehyde
Succindialdehyde**

(b) Tested in 10% propylene glycol solution

Formaldehyde
m-Hydroxybenzaldehyde
5-Nitrosalicylaldehyde

Succinaldehyde
1,2,5,6-Tetrahydrobenzaldehyde
(25% propylene glycol)

(2) *Recovery of 40-59 per cent—moderate activity*

(a) Tested in water solution

Acetaldol*
2,3-Dimethoxybenzaldehyde**

(3) *Recovery of 60-79 per cent—slight activity*

(a) Tested in water solution

β -Ethoxypropionaldehyde
Furfural*

Glyoxal

(b) Tested in 10% propylene glycol solution

Aldol*
Benzaldehyde (25% propylene glycol)
n-Butyraldehyde
2-Carboxy-3,4-dimethoxybenzaldehyde*
5-Chloro-2-hydroxybenzaldehyde
O-Chloro-benzaldehyde
 α -Campholenaldehyde
3,5-Dibromosalicylaldehyde
 β -Ethoxy propionaldehyde (15%
propylene glycol)

2-Ethyl-2-hexanal
Gossypol
2-(Hydroxymethyl)-2-methyl-
butyraldehyde
p-Hydroxybenzaldehyde (20%
propylene glycol)
p-isoPropyl benzaldehyde
Salicylaldehyde
Tolyl aldehyde

(4) *Recovery of over 80 per cent—little or no activity*

(a) Tested in water solution

N-aldopiperazine**
L-(+)Arabinose
 α -Campholenaldehyde
p-Dimethylaminobenzaldehyde**
2,4-Dimethyl-3-formyl-carbethoxy-
pyrrole**
Ethylbutyraldehyde**
2-Ethylhexaldehyde**
Ethyl vanillin**
Furfural*
m-Formyl benzene sulphonic acid**
D-(+)Glucosamine HCl**
Glyceraldehyde*
 α -Hydroxyadipaldehyde*
p-Hydroxybenzaldehyde
2-Carboxy-2,4-dimethoxybenzaldehyde
2,3-Dihydro-2-formal-1,4-pyran**

2,4-Dihydroxybenzaldehyde**
4-Hydroxybenzaldehyde-3-sulphonic
acid*
*iso*Butyraldehyde*
D-(-)Lyxose**
Phthaldehydic acid**
Piperonal**
Propionaldehyde**
D-(-)Ribose
o-Sulphobenzaldehyde
Tiglaldehyde
dl-4,6,6-Trimethyl-3-cyclohexene-1-
carboxyaldehyde
Tolyl aldehyde**
O-Vanillin**
Veratraldehyde**

TABLE 1—continued

(b) Tested in 10% propylene glycol solution

Acetaldo	A-Hydroxyadipaldehyde
4-Acetamidobenzaldehyde	<i>p</i> -Hydroxybenzaldehyde
Anisaldehyde	2-Hydroxy-5-bromobenzaldehyde
L-(+)-Arabinose	<i>m</i> -Hydroxybenzaldehyde carbanilate
α -Campholene aldehyde	<i>iso</i> Butyraldehyde*
Cinnamaldehyde	<i>iso</i> Valeraldehyde (20% pg)
Citronellal	4-Methoxybenzaldehyde-3-sulphonate
2,4-Dichlorobenzaldehyde	α -Methylcinnamaldehyde
Dicrotonaldehyde	<i>l</i> -Naphthaldehyde
<i>p</i> -Diethylaminobenzaldehyde	<i>m</i> -Nitrobenzaldehyde (25% pg)
3,4-Diethoxybenzaldehyde	<i>p</i> -Nitrobenzaldehyde (25% pg)
2,3-Dihydro-2-formal-1,4-pyran	5-Nitrosalicylaldehyde
2,3-Dimethoxybenzaldehyde	Pentachlorobenzaldehyde
<i>o</i> -Ethoxybenzaldehyde	α -Pentylcinnamaldehyde
3-Ethoxy-4-hydroxybenzaldehyde	Phenylacetaldehyde
α -Ethylcaproaldehyde	Piperonal
2-Furanacrolein	Tiglaldehyde
Furfural*	1,2,5,6-Tetrahydrobenzaldehyde
3-Formylphenyl-N-phenylcarbamate	2,4,6-Trimethyl-1,2,5,6-tetrahydrobenzaldehyde
D-(+)-Glucosamine**	Tolyl aldehyde
Glyoxal	O-Vanillin
<i>n</i> -Heptaldehyde	<i>n</i> -Veratraldehyde
Hydrocinnamaldehyde	

Part B—Ketones (RCOR)

(1) Recovery of 0-39 per cent—high activity

(a) Tested in water solution

2,4'-Dihydroxybenzophenone
Pyruvic aldehyde

Rose Bengal*

(b) Tested in aqueous propylene glycol

2-(Dibutylamino)-ethyl phenyl ketone**
2,4'-Dihydroxybenzophenone (62B)
2,5-Dimethyl-*p*-quinone (30% pg)

*iso*Phorone
p-Quinone
dl-Verbenone

(2) Recovery of 40-59 per cent—moderate activity

(a) Tested in water solution

Chloranil**
2,5-Dihydroxy-*p*-benzoquinone*

4-Methylcyclohexanone*
2,4-Pentanedione*

(b) Tested in aqueous propylene glycol

2-Benzoyl-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one
2,4-Butanedione-2-methoxine
m-Hydroxyacetophenone (62B)
5-Hydroxy-2-hydroxymethyl-1,4-pyrone**

Oleone
Phenoxy-2-propanone
Triketohydrindene hydrate
d-Verbenone

(3) Recovery of 60-79 per cent—slight activity

(a) Tested in water solution

Acetone (10%)
Acetylacetone*
2,3-Butanedione-2-oxime*
Dehydroacetic acid
Dehydroacetic acid oxime
4-(5-nitro-2-furyl)-3-buten-2-one

p-Hydroxypropiophenone**
4-(5-nitro-2-furyl)-3-buten-2-one
1-Phenacylpyridinium bromide
Pyruvic aldehyde*
4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione**

TABLE 1—continued

(b) Tested in aqueous propylene glycol

α -acetyl- α -(1-hydroxyethylidene)- β -oxo-glutaric acid, delta lactone
 1-Amino-4-hydroxyanthraquinone
 2-Amino-1,4-naphthoquinone imine
 2-Aminopropiophenone
 2,3-Butanedione, monoxime
 3-Chloro-4'-hydroxy-3'-methoxy-propiophenone
 Butyrophenone
 cyclopentanone
 Diacetone alcohol*
 Dimethoxybenzophenone
 5,5-Dimethyl-1,3-cyclohexanedione*
 2,6-Dimethyl-1,4-pyrone*
 2,5-Dimorpholino-1,4-cyclohexanedione
 1,3-Diphenyl-2-propanone (25% pg)
 3-Heptanone

Laurone
 Mesityl oxide*
p-Methoxypropiofenone
p-Methylacetophenone
 3-Methyl-2-butanone
 4-Methyl-2-pentanone
 6-Methyl-1,2 H-pran-2,4-(3H)-dione
 1,2-Naphthoquinone-4-sulphonic acid
 Naphtho-1,2-quinonyl-4-sulphonic acid
 Naphtho-1,4-quinonyl-2-sulphonic acid
 Phenyl-2-propanone
 3-Pentanone*
 Quercetin
 L-Sorbose*
 Pyruvic aldehyde
 Sodium benzenoneindophenol

(4) Recovery of over 80 per cent—little or no activity

(a) Tested in aqueous solution

Acetocetanilide
 Acetylacetone**
 l-Adrenochrome
 Alizarin Red S*
 Alloxan**
 Aminoacetophenone**
 Benzoin
 Benzophenone**
p-Bromobenzophenone**
 3-Bromocamphor**
 2-Butyl-cyclohexanone**
 Chelidonic acid*
p-Chloroacetophenone**
 Chloroanilic acid**
p-Chlorobenzophenone**
 α -Chloroisnitrosoacetophenone**
p-Chloropropiophenone
 Dehydroacetic acid**
 1-Desoxy-1-(1-piperidyl)D-fructose**
 Diacetoethylenediacetamide*
 Diacetone alcohol*
 Diethylethylidene bisacetoacetate**
 1,5-Di-2-furyl-3-pentanone**
 Dimedone
 5,6-Dimethoxy-1-cyanoisobenzofuran-3-one**
 5,6-Dimethoxyhydrindone**
 1,2-Dipiperidino-2-11-dodecanedione
 Ethyl acetonedicarboxylate**
 Ethyl 2-(*n*-butyl)-3,3-ketobutyrate*
 Ethyl 2,2-di(*n*-butyl)-3-ketobutyrate
 Ethyl 2-(*n*-dodecyl)-3-ketobutyrate
 Furfuralacetone**
 2-Furyl methyl ketone
 1-(2-Furyl)-1-propanone**
 Glucuronolactone*
 Hexahydrolupulone**
 2,5-Hexanedione
 Hexachloro-3,5-cyclohexadion-1-one**

O-Hydroxyacetophenone**
p-Hydroxybenzophenone**
 2-Hydroxy-2-methyl-2-cyclopenten-1-one*
 3-(1-Hydroxy-1-methylethyl)-6-oxoheptanoic acid, gamma lactone
 3-Hydroxy-2-methyl-1,4-pyrone**
 Isatin**
 isoPhorone
 Kojic acid**
 Leocillinic acid*
 5-Methylisatin**
 2-Methyl-1,4-naphthoquinone**
 Methyl-2-phenyl-4-quinolyl ketone**
 Methyl-3-thionaphthyl ketone**
 Morin
 1,2-Naphthoquinone**
 1-(5-Nitro-2-furyl)-1-propanone
 Oxalacetic acid**
 2-Pentanone*
 Phenacyl acetate**
p-Phenacyl phenacyl bromide**
 Phenyl- β -pyridyl ketone HCl*
 β -Piperidinoethyl-2-thienyl ketone HCl
 Pyruvic acid**
 Santonin**
 Sodium anthraquinone- β -sulphonate**
 Sodium benzenoneindophenol**
 Sodium 2,6-dichlorobenzeneoneindo-3'-chlorophenol**
 Sodium magnesium chlorophyll**
 Sodium rhodizone**
 4-(Tetrahydro-2-furyl)-2-butanone**
 2,2,6,6-Tetrakis(hydroxymethyl)-cyclohexanone*
 Thymoquinone**
 Usnic acid**
 dl-Verbenone
 Vitamin K

TABLE I—continued

(4) Recovery of over 80 per cent—little or no activity

(a) Tested in aqueous propylene glycol (10%)

<i>Cis</i> (and <i>trans</i>)- α -acetyl-2-furnanacrylic acid ethyl ester	Hesperidin
Acetonyl acetate*	3-Hexene-2,5-dione
<i>p</i> -Acetyl phenoxyacetic acid	5-Hexene-2-one
<i>p</i> -Amino benzophenone	2-(4-Hydroxybenzoyl)benzoic acid
4-Amino-4'-chlorobenzophenone	4-Hydroxycyclohexenone
4-Amino-4'-methylbenzophenone	3-Hydroxyflavone
2-Amino-1,4-naphthoquinonimine	2-Hydroxy-9-fluorenone
<i>p</i> -Aminopropiophenone	2-Hydroxy-3-methyl-cyclopenten-1-one*
<i>p</i> -Anisoin	3-Hydroxy-3-methyl-2-butanone*
<i>p</i> -Anisyl styryl ketone	4-(<i>o</i> -Hydroxyphenyl)-3-buten-2-one
<i>d</i> -isoAscorbic acid*	1,3-Indanedione
<i>p</i> -Benzoylphenoxyacetic acid	1-Indanone
(2,2-Biindan)-1,1',3,3'-tetrone	Kojic acid
<i>p</i> -Bromoacetophenone	Laurophenone
2,3-Butanedione*	Levulinic acid*
2-Butanone*	7H-6-Methoxycycloheptabenzene-7-one
<i>d</i> -Camphor	2-Methyl-1,4-naphthoquinone
<i>d</i> -10-Camphor sulphonic acid*	3-(<i>p</i> -Methyloctyl)-3-hydroxy-1,4-naphthoquinone
Carbanilic acid derivation of 2,3-butanedione-2-oxime	2,6-Bis(6-methyl-4-oxo-2- γ -puranyl-methyl)-4-pyrone
Chalcone	1-Methyl-4-piperidone HCl**
2-Chloromethyl-5-hydroxy-1,4-pyrone	Mesityl oxide*
<i>p</i> -Chloropropiophenone	1,4-Naphthoquinone
cycloHexanone*	<i>o</i> -Nitrophenyl pyruvic acid
Diacetoethylenediacetamide	2,3-Octanedione
Diacetone alcohol*	2-Octanone
Diacetyl*	N-(9-oxo-2-fluorenyl)formamide
Diacetyl monoxime*	3,3'-(9-oxo-2-fluorenylimino)bispropionamide
2,4'-Dichlorobenzophenone	1-Phenyl-1,3-butanedione
2,5-Dichloro- <i>p</i> -benzophenone	4-Phenyl-3-buten-2-one
2,6-Dichloroquinone	1-Phenyl-2,2-propanedione
4,4'-Dihydroxybenzophenone	Piperonyloin
10,11-Dihydroxy-9,12-dioxo-octadecanoic acid	2-Pivaloyl-1,3-indandione
3',4'-Dihydroxy-2-(3-phenylpropyl-amino)-acetophenone HCl	Propiophenone
Dihydro-2,2,5,5-tetramethyl-3-(2H)-furanone*	Pulegone
2,6-Dimethyl-4-heptanone	Quercetin
Dimethyl pyrone	Rutin**
9,12-Dioxo-10-octadecenoic acid	Senecioic acid ester with DL-2-allyl-4-hydroxy-3-methyl-2-cyclopentene-1-one
9,12-Dioxo-octadecanoic acid	Sodium benzenoneindophenol
10,11-Epoxy-9,12-dioxo-octadecanoic acid	Stearone
Ethyl acetoacetate*	1,2,3,4-Tetrahydro-7-methoxy-9-acridanone
Ethyl phenylglyoxylate	2,3,4-Trihydroxydecanophenone
Fenchone	2,3,5-Trimethylquinone
Furoin (25% pg)	2,3,5-Trimethylquinone-6- β -thiopropionic acid
4-(2-furyl)-3-buten-2-one (25% pg)	2-Undecanone
4-(2-furyl)-2-butanone	Valerophenone
2-Furylphenyl ketone	<i>dl</i> - <i>cis</i> Verbanone
2-Heptadecanone	Xanthone
2-Heptanone	Zingerone
4-Heptanone	

TABLE 1—continued

Part C—Organic acids or salts (RCOOH or RCOOM)

(1) Recovery of 0–39 per cent—high activity

(a) Tested in aqueous solution

Bromoacetic**

 α -Bromopropionic*

Iodoacetic**

Mucochloric

(b) Tested in 10% aqueous propylene glycol

Bromoacetic

 α -Bromopropionic2-Butyl- α -chlorophenoxyacetic

(2) Recovery of 40–59 per cent—moderate activity

(a) Tested in aqueous solution

Cholic**

2-Ethylbutyric**

Perfluorobutyric**

1,2,3-Propanetricarboxylic**

Sodium N-lauroyl sarcosinate*

(b) Tested in aqueous propylene glycol, 10%

cycloHexanebutyric

2-cycloHexene-1-caproic

2-cycloPentene-1-valeric

3,5-Di-iodo-2-hydroxybenzoic

N-(Hydroxyethyl)- β -alanine

5-Iodosalicylic acid acetate (20% pg)

Lactic acid chloroacetate*

(3) Recovery 60–79 per cent—slight activity

(a) Tested in aqueous solution

Acetyl-*p*-aminobenzoic** ι -Alanine** β -Bromopropionic*

5-Bromosalicylic acid acetate

N-Butyl maleamic

o-Chlorophenoxyacetic

Cyanoacetic**

2,4-Dichlorophenoxyacetic**

Diglycolic**

3,5-Di-iodosalicylic

Hexachloroendomethylene-tetrahydro-
phthalic acid, *cis* isomer**

Hippuric*

N-Hydroxyethyliminodiacetic**

Indole-3-acetic**

l-Naphthalene acetic**

DL-Serine**

Sodium ricinoleate**

2,4,6-Trihydroxy-*m*-toluic*

Versene T*

(b) Tested in aqueous propylene glycol

 α -Acetyl- α -(1-hydroxyethylidene)- β -oxo-
glutaric acid, delta lactone

3-Amino-5-iodobenzoic

2-Amino-3,5-di-iodobenzoic*

 μ -Amino-*n*-caproic* (25% pg)N²-Benzyl-N-butyl-DL-asparagineBis(carboxymethyl)mercaptopalbenz-
aldehyde*Bis(carboxymethyl)mercaptopal-2,4-
dichlorobenzaldehyde**

5-Bromosalicylic

Butenylphenoxyacetic

o-(2-Butenyl)phenoxyacetic5-*tert*.-Butyl-6-hydroxy-*m*-anisic*p*-*tert*.-Butylphenoxyacetic*n*-Butyric**n*-Caproic α -Carboxyethyl-N-3-Chlorophenyl-
carbamate*

2-Carboxy-3,4-dimethoxybenzaldehyde

 α -Carboxyethyl-N-phenylcarbamate*

(3-Chlorophenethyl)malonic

cis-4-cycloHexene-1,2-dicarboxylic*2- β -Dibromobutyric

N,N-Dibutyl lactamide adipate

2,5-Dihydro-3-methyl-5-oxo-2-
furanacetic

3,5-Di-iodosalicylic (30% pg)

Diphenic*

2-Ethylhexylmaleate*

2-Furanacrylic

Gallic

Glycolic*

N-Hydroxyethylethylene diamine

triacetic*

(4-Hydroxy-3-methoxyphenyl)-2-
thiopyruvic*p*-Hydroxyphenylacetic*

2-Imino-4-oxo-5-thiazolidineacetic

3-Indole propionic

5-Iodosalicylic

Lactic acid acetate

DL-Mandelic

5-Phenylsalicylic

5-Sulphosalicylic*

Syringic

2,3,5,6-Tetrachlorophenoxyacetic

TABLE 1—continued

(4) Recovery of over 80 per cent—little or no activity

(a) Tested in aqueous solution

Acetic*	9,10-Dihydroxystearic**
3-Acetyl aminobenzoic*	Di-iodotyrosine**
Acetyl- <i>dl</i> -methionine**	3,4-Dimethoxycinnamic**
Acetylsalicylic*	<i>p</i> -Dimethylaminocinnamic**
Acetyl- <i>dl</i> -tryptophane**	Di(methyl "Cellosolve") maleate*
D-Alanine**	1,2,2-Dimethyl-1,3-cyclobutane diacetic acid*
3-Aminobenzoic*	N-N'-Dimethylethylene diamine acetic**
<i>p</i> -Aminobenzoic*	Ethylenediamine tetra acetic**
<i>dl</i> -2-Aminobutyric*	(Ethylenedioxy) diacetic*
<i>p</i> -Aminobenzoyl glutamic**	Fencholic**
<i>p</i> -Amino hippuric*	Ferulic**
<i>dl</i> -2-Amino- <i>n</i> -valeric**	Fluoroacetate*
Anthranilic**	Sodium formate**
Asparagine**	DL-2-Furanserine*
<i>l</i> -Aspartic**	Furoic**
Benzilic**	Furylacrylic**
Benzoic**	Glucosaminic**
N-Benzoyl- β -3,4-dimethoxyphenyl(<i>dl</i>)- α -alanine**	L-Glutamine**
DL-3-Benzylamino-succinamic	Glycine**
Betaine anhydrous*	Guanidoacetic**
α -Bromo- ϵ -benzoylamino caproic**	(Guanidino-oxy)acetic
β -Bromobutyric*	L-Histidine HCl*
5-Bromo-3-hydroxybutyric*	DL-Homocystine**
Bromostearic*	2-Hydroxybutyric*
1,2,3,4-Butanetetra-carboxylic**	<i>p</i> -Hydroxycinnamic**
2-(<i>p</i> -tert.-Butyl-phenoxy)propionic**	<i>dl</i> -Hydroxycitric*
Calcium levulinate*	(4-Hydroxy-3-methoxyphenyl)-pyruvic acid oxime
Calcium tartrate**	β -Hydroxynaphthoic**
Cetyl betaine chloride*	<i>p</i> -Hydroxyphenoxyacetic**
Chelidonic**	N-(<i>p</i> -Hydroxyphenyl)glycine**
2-Chloro-4-aminobenzoic**	L-Hydroxyproline**
<i>p</i> -Chlorophenoxyacetic	2-Imino-3-benzothiazoline acetic**
4-Chlorophenoxyacetic**	Iminodiacetic*
Chloroethyl maleate**	γ -Indole-3- <i>n</i> -butyric**
α -(<i>p</i> -Chlorophenoxy)propionic**	<i>iso</i> Nicotinic**
α -(<i>o</i> -Chlorophenoxy)propionic**	β -Iodopropionic*
L- β -Chloro- α -aminopropionic*	5-Iodosalicylic acetate**
Chloroacetic**	DL- <i>iso</i> Leucine**
<i>p</i> -Chlorobenzoic	2-Keto-D-glucuronate**
5-Chloro-2-furoic	Kynurenic**
Citrazinic**	Levulinic**
Citric*	L-(--)-Leucine**
DL-Citrulline**	DL-Lysine HCl*
<i>o</i> -Cresotinic**	Maleamic**
cycloHexane acetic*	Maleic*
β -cycloPentane-propionic*	Malonic**
L-(--)-Cystine**	DL-Methionine**
DL-Desthiobiotin**	DL-2-Methylglutamic*
L-Diaminopropionic acid dehydrochloride**	5-Methyl-3-pyridylacetic**
α , β -Dibromosuccinic*	6-Methyltryptophan
Dichloroacetic**	Nicotinic**
2,4-Dichlorophenoxy acetic**	<i>p</i> -Nitrobenzoic**
2,2-Dichloropropionic*	3-Nitrophthalic**
3,5-Dihydroxybenzoic*	DL-Norleucine**
N,N-Di(2-hydroxyethyl)glycine*	DL-Norvaline**
2,6-Dihydroxy-isonicotinic**	DL-Ornithine**
3,4-dihydroxyphenyl acetic**	Orotic**
<i>dl</i> - β -(3,4-Dihydroxyphenyl)alanine	Oxalacetic**

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TABLE I—continued

(a) Tested in aqueous solution—continued

2,2'-Oxydiacetic**	Sodium magnesium chlorophyll**
Perfluoroadipic**	Sodium salicylate**
Perfluorohexanoic**	Succinic**
Perfluorooctanoic**	Tannic**
Perfluoropropionic**	Tartaric**
Perfluorosuccinic**	6-[2-Tetrahydrothiazole-4-one]-
Phenylacetic*	caproic**
DL-Phenylalanine**	Thioglycolic**
N-Phenylglycine*	DL-Threonine*
DL-β-Phenyl-lactic**	Trichloroacetic*
3-Phenylsalicylic	2,4,5-Trichlorophenoxy acetic
Phthaldehydic**	Trifluoroacetic**
Potassium sodium tartrate**	Trimethylamine- α,α,α -tricarboxylic*
5-Pyrimidinecarboxylic acid, 1,2,3,4-	Trimesic
tetrahydro-2,4-dioxo	DL-Tryptophane**
Pyruvic acid**	DL-Tyrosine
Pyruvic acid oxime**	DL-Valine**
β-Resorcylic	Vanillic**
Sarcosine*	Versene Fe ₃ *
Sodium glycocholate**	

(b) Tested in 10% propylene glycol solution

2-Acetamido-3-methylcrotonic acid**	7-Chloro-4-hydroxy-3-quinoline
N-Acetylthranilic acid	carboxylic
N-Acetyl-3-mercapto-valine*	2-Chloro-3-methylbutyric
p-Acetylphenoxyacetic	3'-Chlorophthalanilic
N-Acetyl-N-phenylglycine	β-Chloropropionic*
p-Aminobenzyl-aminobenzoic	o-Chlorophenoxyacetic
DL-2-Amino-n-caproic	4-Chlorophenylacetic
N-p-[(2-Amino-4-hydroxy-6-pteridyl- methyl)methylaminobenzoyl- 1-glutamic	4-Chloro-o-teloxycetic
DL-2-Aminophenyl acetic	3,5-Diaminobenzoic acid
Amino-7-resorcylic	10,11-Dihydroxy-9,12-dioxo- octadecanoic
5-Aminosalicilic	9,10-Dihydroxy stearic
o-Anisic** (tested in Dowanol 62B)	9,12-Dioxo-octadecanoic
p-Anisic** (tested in Dowanol 62B)	2,4-Dimethoxybenzoic
DL-Asparagine*	(3,4-Dimethoxyphenyl)acetic
α-Benzamido-4-carboxy-2-thiazolidine	10,11-Epoxy-9,12-dioxo-octadecanoic
acetic, α-ethyl ester	o-Ethoxybenzoic
2H-1,4-Benzothiazine-2,2-diacetic-3,4-	Ethylene diamine nitrilotriacetic
dihydro-3-oxo	2-Ethylhexanoic
p-Benzoylphenoxyacetic	Ferulic acid acetate
Benzylmalonic*	N-Formyl-D-leucine
3-(Benzylthio)-D-valine HCl*	Furfurylidenemalonic
Bilirubin	Gentisic**
2-Bromobutyric*	Gentisic acid, 5-acetate
N-(N'-α-bromopropionyl)glycyl-	Glycylglycylglycylglycylglycine
glycine*	Gluconic*
5-Bromosalicylic acid acetate (tested in	n-Heptanoic
Dowanol 62B)	Histidine*
3-tert.-butylgentisic	Hydantoic**
p-tert.-Butylphenoxyacetic	2-Hydroxy-m-anisic
2-Carboxyethyl-N-phenyldithio-	6-Hydroxy-m-anisic**
carbamate	2-(4-Hydroxybenzoyl)benzoic
5-Chloroanthranilic	1-Hydroxycyclohexane carboxylic
2-Chlorobutyric	4-Hydroxyisophthalic
2-(2-Chloroethoxy)ethyl fumarate*	3'-Hydroxyphthalanilic
2-(2-Chloroethoxy)ethyl maleate*	4'-Hydroxyphthalanilic
p-Chlorohippuric**	Imidazole-4,5-dicarboxylic
	3-Indole butyric

TABLE I—continued

(b) Tested in 10% propylene glycol solution—continued

5-Iodosalicylic	2-Phenyl-γ-thiohydantoic
5-Iodosalicylic acid acetate (tested in Dowanol 62B)	2-Phenylthiazolidene-4-carboxylic
<i>o</i> -Iodobenzoic	L-(−)Proline*
5- <i>iso</i> Propyl-2-methyl-phthalanilic	Propionic
Levulinic*	Salicylic
3-Mercapto-DL-valine**	Sulphoacetic
N-Methyl-N-methyl ester betaine octadecylglycine	Sorbic
6-Methylpantothenate**	Succinyl indoline
Mucic	Sulphosalicylic*
<i>m</i> -Nitrohippuric**	2-Sulpho-4'-(2-thiazolylsulphamoyl)-succinanilic
<i>p</i> -Nitrophenylisovaleric acid*	Tetrafluorosuccinic
2'-Nitrophthalanilic	Tetrasodium-N-(1,2-dicarboxyethyl)-N-octadecylsulphosuccinamate*
3'-Nitrophthalanilic	<i>d</i> -thionine*
4'-Nitrophthalanilic	2,3,5(2,3,6 or 2,4,5)-Trihydroxybenzoic
Octadecyl-β-alanine	Trihydroxyterephthalic acid, 2,3,5-
2-Oxo-1,2-benzopyran-3-carboxylic	3,4,5-Trimethoxy benzoic
Phenacetic	2,3,5-Triethylquinone-6-β-thiopropionic
Phenoxyacetic**	Versene regular**
(2-Phenylacetamido)malonic monoethyl ester*	

cent. Absence of inhibitory action would be indicated by recoveries in the 100 per cent range which would show complete recovery of power to produce acid. Low recoveries indicate promising compounds which do not permit recovery of glycolytic action.

RESULTS

Inhibitory aldehydes and related structures are presented in Fig. 1. The simplest aldehyde, formaldehyde, is one of the most inhibitory, but the action of the aldehyde group falls off rapidly with increasing chain length, since propionaldehyde is only moderately inhibitory and butyraldehyde is scarcely active. Among the inactive aldehydes that are homologues of formaldehyde are tigraldehyde, isobutyraldehyde, valeraldehyde, α-ethyl-butyraldehyde, heptaldehyde, α-ethyl-caproaldehyde, 2-ethyl-2-heptaldehyde and citronellal. These compounds are listed in Table 1, along with all other compounds tested in this investigation. The simplest dialdehyde, glyoxal, is completely without effect, whereas the four-carbon dialdehyde, succinaldehyde, is the most inhibitory substance of those presented in Fig. 1. No other dialdehydes were on hand for comparison. The methyl derivative of glyoxal, pyruvic aldehyde, is inhibitory, but only when tested in water. The same compound tested in 10% propylene glycol shows a recovery of 69 per cent. A simple hydroxy aldehyde, aldol, shows borderline inhibition, but the simplest dihydroxyaldehyde, glyceraldehyde, appears to be completely ineffective. Aldol also resembles pyruvic aldehyde in showing its best effect in water solution; when tested in 10% propylene glycol the recovery was 90 per cent. The only remaining acyclic inhibitory aldehyde is mucochloric acid. It has no related structure except chloroacetaldehyde, which was inhibitory in the initial test; a re-test with a partially decomposed sample of chloroacetaldehyde failed to substantiate the inhibitory action.

The next two inhibitory structures (Fig. 1) are phenol derivatives. These may owe their activity to the presence of phenolic hydroxyl groups. The first, *m*-hydroxy benzaldehyde, is inactive in the usual concentration of propylene glycol, as are the related compounds, salicylaldehyde and 3,4-dihydroxybenzaldehyde. The 5-nitrosalicylaldehyde is more active than either of two halogen derivatives of salicylaldehyde, which suggests that inhibition is related to the nitro group rather than the aldehyde portion of the molecule.

The activity of 3-cyclohexene-1-carboxyaldehyde indicates that the aldehyde group can impart activity to some structures in the absence of any other functional group, but 25 per cent propylene glycol is necessary as a solvent. The analogous benzaldehyde is not inhibitory and the 2,4,6-tri-

methyl and 4,6,6-trimethyl derivatives of *cyclohexene-1-carboxaldehyde* show less inhibitory action, perhaps because of lower solubility. This incidence of activity (including chloracetaldehyde) amounts to 8 per cent for the approximately 100 aldehydes studied.

Inhibitory ketones and structures related to these are presented in Fig. 2. The keto-aldehyde, pyruvic aldehyde, is repeated and compared with acetone and 2,3-butanedione, neither of which had any essential inhibitory action. Note that acetone was tested at 10% concentration. The inhibitory action of pyruvic aldehyde is apparently attributable to the aldehyde group rather than to the ketone. The inhibitory action of 2,4-pentanedione is borderline. Other similar structures, 2,5-hexanedione, 3-hexene-2,5-dione, and 2,3-octanedione are inactive. The next compound is a

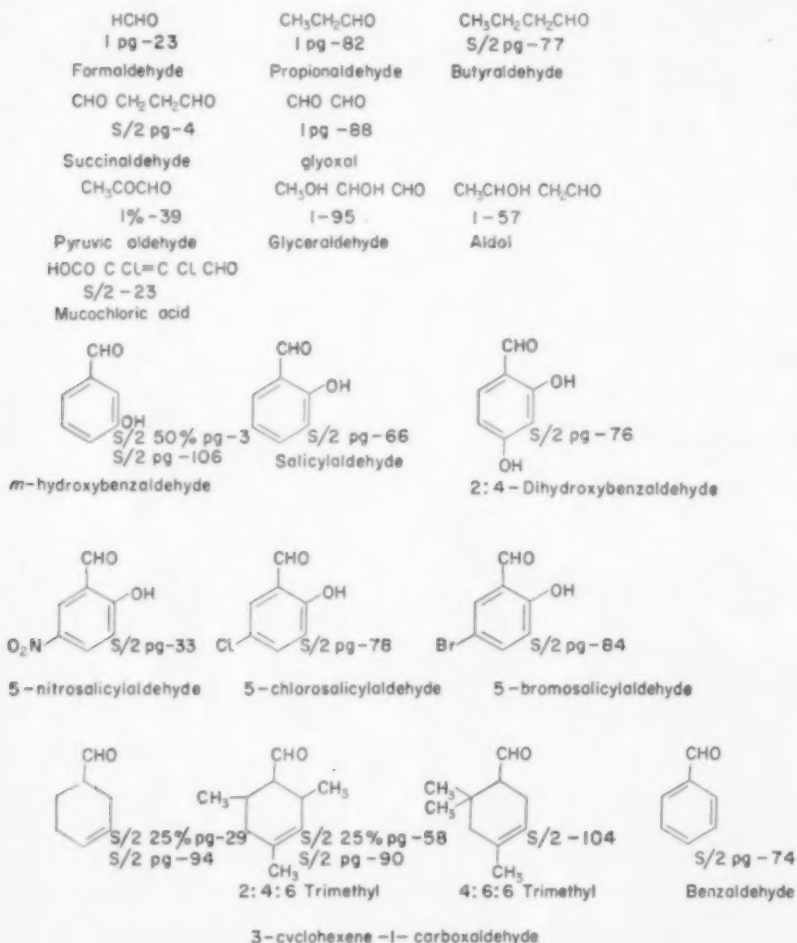


FIG. 1. Inhibitory aldehydes and related structures.

long chain ketone, "oleone", which possesses inhibitory action of more than 40 per cent. The corresponding substance without double bonds, di-steryl ketone, is not inhibitory. A chemical with a shorter chain, dilauryl ketone, and mesityl oxide, a short chain unsaturated ketone, both show slight inhibitory action. There are many additional related substances which had no inhibitory action. These include 2-butanone, 2-pentanone, 2-heptanone, 2-octanone, 2,6-dimethyl-4-heptanone,

2-undecanone, and 2-heptadecanone. Thus there are negative tests on ketones with chain lengths ranging from three to seventeen carbon atoms (except for mesityl oxide), then two inhibitory compounds with twenty-five and thirty-seven carbon atoms and an inactive compound with thirty-seven carbon atoms. There is a high degree of specificity for glycolysis inhibition among higher ketones.

The 2-methoxime of 2,3-butanedione is inhibitory and the monoxime of the same compound shows some slight action. Since the original diketone does not possess inhibitory action, the effect is probably attributable to the oxime rather than to the ketone.

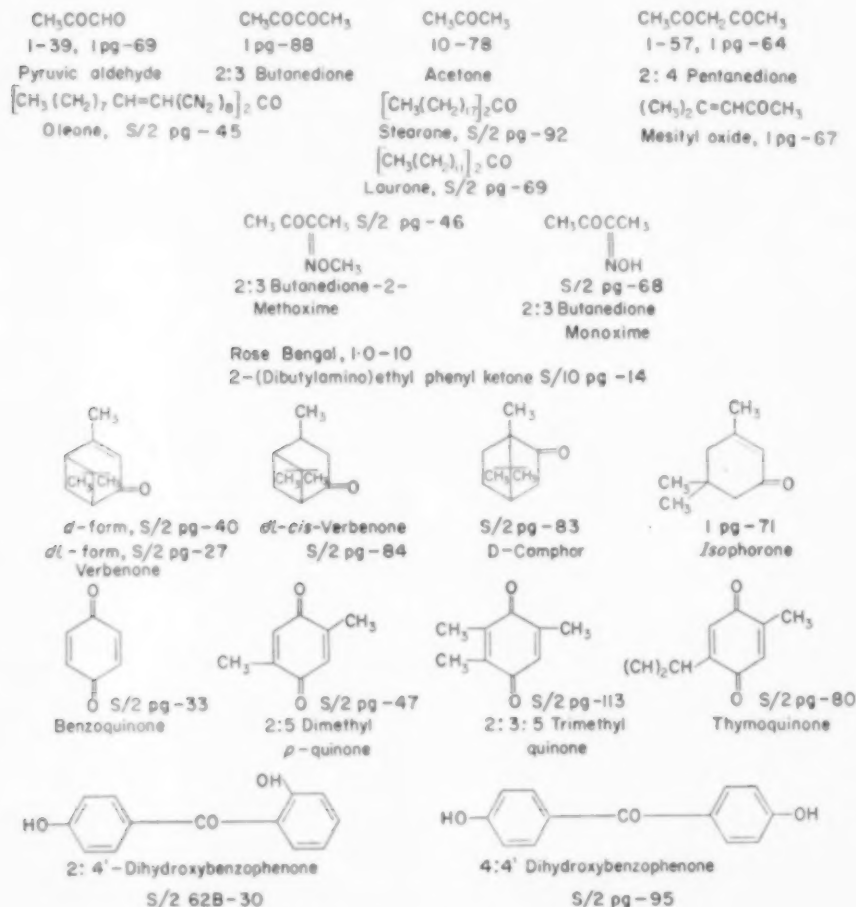


FIG. 2. Inhibitory ketones and related structures.

The next chemical is Rose Bengal, a complicated structure involving three alicyclic rings with chlorine and iodine substitution and ether, phenol, ketone and acid groupings. No compounds are available which are essentially similar, and the complexity of the structure makes it unlikely its inhibitory action is attributable solely to the ketone group. The structure of the next inhibitory compound, 2-dibutylaminoethylphenyl ketone, is not reproduced for a similar reason. There are no similar dibutylamines lacking only the keto group, but there is a dibutylamine in which the inhibitory activity is attributable to the nitrogen rather than to another constituent.

The next group of compounds show unusual degree of specificity. The D and DL forms of verbenone are inhibitory whereas the DL *cis* form, lacking the double bond, is not inhibitory. D-Camphor is not inhibitory; it has a similar structure but lacks a double bond. *iso*Phorone lacks the bridged ring and activity. Fenchone was also not inhibitory; this compound is the 3,3-dimethyl analogue of 4-camphor, lacking its two central methyl groupings. Other evidence suggests that the inhibitory effect is probably attributable to the bridged unsaturated ring, rather than to the ketone itself. Verbenone is inactive when tested in water solution, and thus the value of the ketone may be in promoting solution of an inhibitory ring structure. In support of this hypothesis is the finding that verbenyl amine is active. Some doubt is cast upon it by the fact that the dimethylamine derivative in the same position is not inhibitory. Perhaps the degree of specificity is so great that no generalizations can be made.

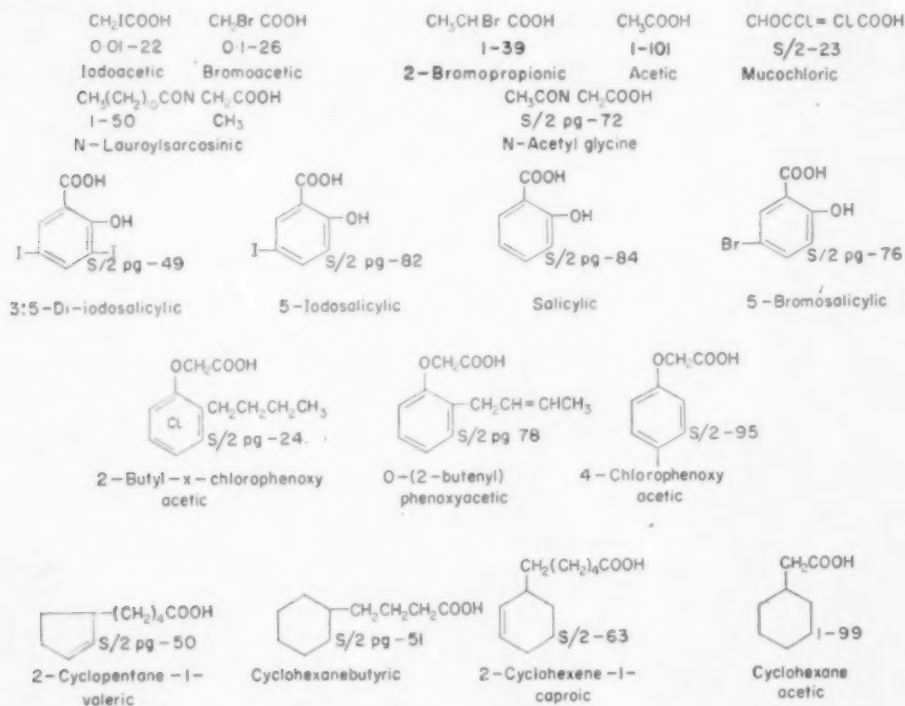


FIG. 3. Inhibitory acids and related structures.

The next comparisons are between the two active substances, benzoquinone and the 2,5-dimethyl-paraquinone, and two inactive substances, 2,3,5-trimethylquinone and thymoquinone. A high degree of specificity is indicated from a comparison of the last two compounds in Fig. 2. Both are diphenols as well as ketones. The 2,4'-dihydroxybenzophenone has been shown to be inhibitory by repeated tests, whereas the 4,4' compound is not inhibitory. The related 2-(4-hydroxybenzoyl)-benzoic acid and benzoin, were both inactive when tested at half saturation in propylene glycol.

In general, it appears that the ketones can only serve as auxiliary groups, perhaps aiding solution of some of the difficultly soluble compounds, or permitting by enolization the formation of active hydroxyl compounds. There is no indication of a general group of active ketones such as may be observed among amides (MANLY and HARGREAVES, 1956) or amines (MANLY, 1954b).

Inhibitory acids and related structures, presented in Fig. 3, are also characterized by a high degree of specificity. The first type consists of those related to the active substance iodoacetate, which is inhibitory in aqueous solution at 0.01% concentration. Bromacetate is inhibitory at ten

times the concentration and bromopropionate is inhibitory at 100 times the concentration. Acetic acid is without effect. Mucochloric acid has already been discussed under aldehydes, but is reproduced here since it may owe its effect to its resemblance to iodoacetate. Also inactive were trichloroacetic, fluoroacetic, propionic, β -chloropropionic, β -bromopropionic and β -iodopropionic acids. Among the inactive butyric acid derivatives are butyric, 2-chlorobutyric, 2- and 3-bromobutyric and 2-chloro-3-methylbutyric acids.

The glycolysis-inhibiting property of sodium N-lauroyl sarcosinate was described first by FOSDICK *et al.* (1953). The most closely related structure tested in this study is acetyl-glycine, which is less active. In addition a considerable number of less related structures have been tried with negative results. These include N-[N'- α -bromopropionyl]-glycyl-glycine, N,N-dibutyl-lactamide adipate and N-acetyl-3-mercaptopalane. No generalizations can be drawn from these structures because of the lack of closely related compounds.

A good example of specificity in glycolysis inhibition can be seen from compounds related to the active substance 3,5-di-iodosalicylic acid. Salicylic acid and its 5-iodo derivatives were both inactive. The 5-bromosalicylic acid has less effect than 3,5-di-iodo compound. Several other salicylic acid derivatives have been tried. These include the 3- and 5-phenyl, 3-methyl, 3-*tert*.-butyl, 5-hydroxy and the 5-carboxy derivative. None of these were active. The 4- and 5-hydroxy derivatives of salicylic acid were tested at lower concentrations and were not inhibitory. None of these findings suggest that the inhibitory action of the 3,5-di-iodosalicylic acid is attributable chiefly to the carboxyl group.

The inhibitory compound 2-butyl- α -chlorophenoxy acetic acid possesses halogen, ether and carboxyl groupings. The ether grouping is rarely inhibitory (MANLY and HARGREAVES, 1957). Closely related compounds are inactive. The 4-chlorophenoxyacetic acid lacks only the butyl grouping and shows no inhibitory effects. The 2-butenyl derivative lacks the halogen and possesses a double bond, and gives slight evidence for inhibitory action. Tests were made on thirteen additional related structures which are not set forth in Fig. 3. Some indication of activity was observed by the *p*-tertiary-butylphenoxyacetic acid and for the 2,3,5,6-tetrachloro derivative of phenoxyacetic acid, but none for the 4-chloro derivative of this substance. The 2-methyl, α -chlorophenoxyacetic acid was also tried without effect. The remainder of the thirteen substances were tried at lower concentrations than normal and the negative findings cannot be used to prove the absence of inhibitory effect. These findings are insufficient to permit a generalization to be made, especially since the activity of 2-butyl- α -chlorophenoxyacetic acid might be attributable to an impurity, but they offer little to indicate that the carboxyl group contributes to the inhibitory action.

The last four structures in Fig. 3 offer direct evidence that the carboxyl group can be inhibitory, because in these compounds it is the only functional group present, involving atoms other than carbon and hydrogen. 2-cyclopentene-1-valeric acid is on the borderline of inhibition and cyclohexene butyric acid has similar inhibitory effect. In a previous report cyclohexene-1-caproic acid was ranked as being more active than either of these two (MANLY and HARGREAVES, 1955), but recent tests have failed to substantiate this. On the other hand, the cyclohexene acetic acid, the most nearly related structure, was not inhibitory when tested at 1% concentration in water. The only related compound tested and not present in Fig. 3 is *cis*-4-cyclohexene-1,2-dicarboxylic acid, which showed recovery of 68 per cent. Apparently it can be concluded that some of the alicyclic carboxylic acids, either with or without one double bond in the ring, possess ability to inhibit glycolysis. Few generalizations can be drawn because of lack of sufficient structures of this type for investigation. There still remains the question as to whether or not the essential requirement for inhibitory action can be attributed to carboxyl or whether the carboxyl merely serves as a means for bringing into solution those ring structures which have the potentiality for inhibition.

DISCUSSION

Several of the compounds which were found to have the property of glycolysis inhibition toward salivary sediment also possess other biological activities. There is an abundance of information concerning a few compounds, and a dearth of literature regarding most. Iodoacetate is capable of reducing caries in experimental animals (MILLER, 1938; MCCLURE and ARNOLD, 1941; POWELL and DALE, 1943; MCCLURE,

1948; LUNDQUIST, 1951), of acting as a glycolysis or enzyme inhibitor (BACQ, 1941; ALDOUS, 1952, and NATTO, 1955), and as a bactericide (WRIGHT, 1938; YANAGITA, 1947; ALDOUS, 1948; FITZGERALD and JORDAN, 1953). The compound probably has little potential value as a dentifrice or food additive because of its toxicity (LUNDQUIST, 1951). Less literature is available on *p*-benzoquinone, but it is known to have bactericidal effects (NASSI, 1946; DONATELLI and DAVOLI, 1946; SCHRAUFSTÄTTER, 1950), and to possess inhibitory properties on certain enzyme systems (KUHN and BEINERT, 1947; MEYERHOF and RANDALL, 1948; ACKERMANN and POTTER, 1949), even though the chemical appears to be incapable of influencing dental caries of experimental animals when it is added to a dry cariogenic ration (GRANADOS, GLAVIND and DAM, 1949). The similar compound, phlorone, is an inhibitor of yeast carboxylase that is less effective than *p*-benzoquinone (KUHN and BEINERT, 1947); another structure, verbenone, has antifungal properties (OSTER and GOLDEN, 1948). The ketoaldehyde, pyruvaldehyde, is an inhibitor of succinic dehydrogenase (KUN, 1950).

The generalization seems to be that some of the active glycolysis inhibitors have a wide spectrum of biological activity. Probably the same generalization would hold for chemicals that are inactive as glycolysis inhibitors. The findings on glycolysis inhibitors should be poorly correlated with any other measures of biological activity because of the requirements which have been set in our screening programme; in addition to possessing one or more of several types of biological activity, these substances must also be able to penetrate thin films containing micro-organisms, and must have an irreversible action.

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THE COMPOSITION AND PROPERTIES OF THE MUCIN CLOT FROM CATTLE SUBMAXILLARY GLANDS*

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Abstract—Mucin clots from cattle submaxillary glands were lyophilized as sodium salts with retention of the viscosity of the extracts and of solubility in water. The viscosity was 25.8 cP in 1% aqueous solution and 4.5 cP in 1% phosphate buffer of pH 7.4 and ionic strength 0.1. The specific rotation was $[\alpha]_D^{25} = -23.2^\circ$ (0.87 per cent in water). The mucin contained about 13 per cent sialic acid, 11 per cent hexosamine (8 per cent galactosamine and 3 per cent glucosamine as the acetyl derivatives) and 75 per cent protein. The molar ratio of galactosamine to sialic acid was unity.

The mucin showed four peaks when subjected to electrophoresis at pH 10 in a phosphate buffer of ionic strength 0.1. The average mobilities were -7.2 , -8.0 , -10.3 and -11.3×10^{-5} cm²/sec per V, respectively, at pH 10. Three components were evident at pH 7.4 and 8.0, and only two at pH 6. The major component had a mobility of -6.3×10^{-5} at pH 7.4.

Two ultracentrifugal components of 2.1 and 2.5 S were evident at pH 7.4. At pH 10, a small more rapidly moving 5.0 S component was also present.

HAMMARSTEN (1888) found that extracts of cattle submaxillary glands deposited a viscous stringy mucin clot in weakly acid solutions and reported the general properties of the clot. Although some later investigations were made of the composition (TANABE, 1939; BLIX, 1940; BLIX, SVENNERHOLM and WERNER, 1952), degradative procedures were often used especially since the purpose was to study specific components such as glycoprotein, polysaccharide or sialic acid (BLIX *et al.* 1956; HEIMER and MEYER, 1956; GOTTSCHALK, 1956, 1957). In one study, the materials in the supernatant from the clot were studied rather than the clot (CURTAIN and PYE, 1955).

This paper presents information on the composition and properties of the clot obtained under conditions such that minimal changes were produced except when desired. Subsequent papers will describe additional work on the fractionation of the clot to components which no longer form clots on mild acidification.

EXPERIMENTAL

Collection and identification of glands. The cattle submaxillary glands were collected through the courtesy of Dr. A. Roth (A. Roth & Co., Phil., Pa.) and Dr. James B. Lesh (Armour Laboratories, Kankakee, Ill.). The glands were frozen immediately after collection and kept frozen until several hours before use.

A gross and histological examination of the glands by Drs. C. Klapper and T. Weatherford was made because some samples of the glands as received did not seem to be properly identified.

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However, since the cells were somewhat damaged by being frozen, they were identified by comparison with several samples obtained from fresh glands. These were obtained from the J. M. Thomason Packing Co., Bessemer, Alabama, through the kindness of Dr. H. E. Harriman.

The submaxillary gland was pale yellow (whereas the parotid gland was pink). The submaxillary glands were surrounded by a definite capsule of connective tissue and could easily be dissected from the surrounding tissues. The average weight of each individual fresh gland was approximately 150 g.

Three small portions, taken from different parts of the fresh glands obtained immediately after death, were fixed in Bouin's solution (picric acid, acetic acid, formaldehyde). Histological sections were stained with haematoxylin-eosin. Microscopic examination revealed that most of the alveoli were of the serous secretory type, similar to those which made up the parotid glands. A small percentage of the alveoli were of the mixed type with a demilune of serous cells surrounding the terminus of the alveolus of the mucous cells.

Conditions for the extraction. The bovine submaxillary glands were thawed, separated from fat and connective tissue as cleanly as possible, and cut into 4-7 mm cubes.

A 40 g portion of the cut glands was extracted successively with seven changes of a phosphate buffer (CLARK and LUBS, 0.01 M, pH 7.0) at 2-4°C for 24 hr each. For extracts 1, 2, 3 and 7, 50 ml of buffer was used; for the others 75 ml was used. Each extract was filtered through four layers of cheese cloth, and the residual glands were freed from the extract as much as possible by squeezing them in the cloth. After the third extraction, the glands were ground in a meat grinder, and the ground glands were treated as above. The first extract was red in colour, but the others were almost colourless.

Each extract was centrifuged at 20,000 r.p.m. (34,850 g) for 30 min. Floating fat particles were removed mechanically with the aid of a spatula and by filtering through a dry cotton cloth. The clear solution was adjusted to pH 3.5 with 0.05 N hydrochloric acid. The resulting clots were removed by centrifugation at 1500 r.p.m. for 15 min. These were dissolved in sodium hydroxide solution of pH 8-9 (see next section). The content of sialic acid and protein in the extracts, clot solutions, and supernatants of clots were determined by the direct Ehrlich (PIGMAN *et al.*, 1958) and Biuret reagent (ROSENTHAL and CUNDIFF, 1956), respectively.

The results are shown in Fig. 1. The ratio of the content of sialic acid to that of protein in the clots fell in the range 0.15-0.20, except for the first and seventh extracts which had ratios of 0.08 and 0.12, respectively. In subsequent work, extracts corresponding to the second through the sixth were used for the preparation of mucin.

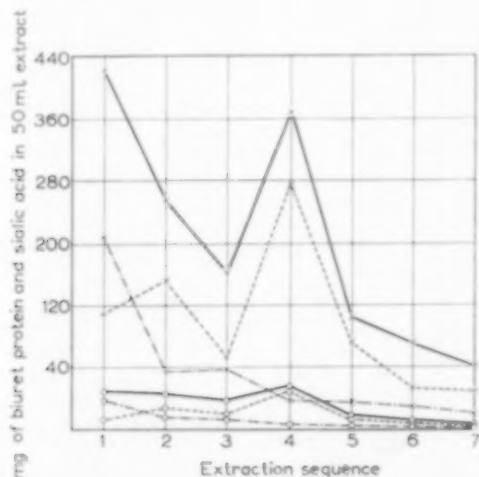


FIG. 1. Distribution of protein and sialic acid in the extracts, mucin clots and supernatants of clots. \times : Biuret protein; \circ : sialic acid. (— Extract; - - - - - mucin clot; — · — · — supernatant).

Preparation of mucin. The extracts obtained in the above fashion from 200 or 250 g of the glands were combined in three groups: (A) pooled second and third extracts; (B) fourth extract; (C) pooled fifth and sixth extracts. They were cleared by centrifugation at 34,850 g and acidified by 0.05 N hydrochloric acid to pH 3.5. The clots were removed by wrapping them around a spatula. They were washed with water, suspended in a 4-5 fold volume of water, and 0.05 N sodium hydroxide was added drop by drop with mechanical stirring, keeping the pH of the solution below 9. After complete dissolution of the clots, the solutions were dialysed with mechanical stirring against two changes of about 50 fold volumes of water for 3 days. All work was carried out at 2-4°C. The dialysates were lyophilized after centrifugation at 34,850 g for 30 min and filtration through a dry cotton cloth.

The mucins obtained from extracts A, B and C were named mucin A, B and C respectively. From 2.5 kg of cattle submaxillary glands, 18 g of A, 6 g of B and 14 g of C, a total of 38 g (as sodium salt) was obtained. The yield was 1.5 per cent of the moist weight of glands or 5.4 per cent of the dry weight of the glands.

Chemical analyses. Total nitrogen was determined by the micro-Kjeldahl method (CLARK, 1943). Sialic acid was measured by the modified direct Ehrlich method (PIGMAN *et al.* 1958). Hexosamine was analyzed by the Elson-Morgan method modified by BOAS (1953). For the measurement of glucosamine and galactosamine separately, the method of GARDELL (1953) was utilized; a 1×38 cm column with Dowex 50 ion exchange resin (W-X8, 200-400 mesh) was used, and a volume of 1.1-1.2 ml of eluate (0.3N HCl) was collected in each test tube on a fraction collector. Proteins were determined by both the Biuret reagent (ROSENTHAL and CUNDIFF, 1956) and the phenol reagent (LOWRY *et al.*, 1951). Total aldose was determined by the anthrone method (HANSON, SCHWARZ and BARKER, 1955), fucose (6-deoxyhexose) was measured by the cysteine-H₂SO₄ method (WINZLER, 1955) and uronic acid by the carbazole reagent (DISCHE, 1955).

The moisture content of solid materials was determined in a Abderhalden apparatus over P₂O₅ at 100°C. For solutions, the salts were removed by dialysis against water after a little 5% EDTA solution had been added inside the cellophane bag. An aliquot was then dried to constant weight at 100°C in an open oven. The ash determination was made by ignition in a platinum crucible. Ether-soluble materials were determined by using the Soxhlet apparatus.

A qualitative test for phosphorus was carried out with ammonium molybdate after ignition of samples with sulphuric acid and nitric acid (KOCH and MARTIN, 1943). For ester sulphate, barium sulphate formation in a slightly acid medium was used after hydrolysis of the samples with 2.4 N hydrochloric acid.

Physical measurements. Viscosities were determined in Cannon-Manning (Ostwald type) viscometers of about 0.2 ml capacity at 30.0°C. The flow times for water in the four viscometers was: No. 100, 90 sec; No. 200, 9.9 sec; No. 300, 3.8 sec; No. 400, <2 sec.

Electrophoretic analyses were made on solutions dialysed in a Cellophane bag for 24-48 hr at 2-4°C against the buffer. A Perkin-Elmer model 38 Tiselius-type

electrophoresis apparatus was used with 6 ml cells. The ascending boundaries were utilized for the calculations of mobility.

Ultracentrifugal analyses were made at room temperature by use of the Spinco Model E analytical ultracentrifuge at 259,700 *g* using a 12 mm cell. Mr. E. Gramling made these measurements.

The buffers used for this work (called phosphate buffers) were: the Miller-Golder sodium phosphate-sodium chloride of ionic strength 0.1 and pH 6.2, 7.4, 7.8 and 8.0; and a Na_2PO_4 (0.0069 M), Na_2HPO_4 (0.0051 M) and NaCl (0.0433 M) solution which after dialysis of samples showed a pH of 10.1–10.8.

RESULTS

A total of about 38 g of a dry colourless product was obtained, divided into three fractions representing different stages of extraction. Mucin A represented the first extractions, mucin B the major intermediate fraction and mucin C the last extractions. These products contained 6.3 per cent moisture, 4.0 per cent ether soluble materials and 1.5 per cent direct ash. Based on qualitative tests, the ash was found to be composed mostly of Na_2CO_3 .

The mucins dissolved completely in water to give clear colourless solutions which were viscous and "stringy" and which gave a mucin clot at pH 3.5. The general composition and some of the properties of these materials are given below. As far as could be ascertained (see below), the isolated materials retained the general mucous characteristics of the extracts, and no indication of degradation of the materials could be found. However, when the original HAMMARSTEN procedure was used and the clots were purified by solution in 0.1 N hydrochloric acid, some of the material became insoluble in each treatment, and the viscosity of the products gradually decreased.

Viscosity measurements

Effect of velocity gradient. The viscosity of a solution of mucin C was measured in four viscometers of widely different rates of flow. The results, given in Table 1, show that the absolute and relative viscosity are practically independent of flow rate.

TABLE 1. VISCOSITIES AT 30.0°C OF SOLUTIONS OF MUCIN C MEASURED IN VISCOMETERS OF DIFFERENT FLOW RATES

Solution	Viscosity in Cannon-Manning viscometer number							
	100		200		300		400	
	Centi-poise	η_r	Centi-poise	η_r	Centi-poise	η_r	Centi-poise	η_r
1% in water	26.0	29.5	25.4	29.8	25.9	30.2	26.4	—
0.5% in phosphate buffer, pH 7.4, ionic strength 0.1	2.4	2.8	2.4	2.8	—	—	—	—
1.0% in same buffer	—	—	—	—	4.45	5.2	4.3	5.1

Effect of the preparatory procedure on the viscosity of the mucin. In order to determine whether clot formation at pH 3.5 and whether lyophilization produced changes in the mucin, the following solutions were prepared and their viscosities were measured: A, 0.5% solution of mucin A in phosphate buffer (pH 7.4, ionic strength 0.1); S₁, pooled centrifuged extract from which mucin A was prepared; S₂, solution of mucin A which was used for the preparation of lyophilized A. The solutions of S₁ and S₂ were dialysed for 24 hr against the phosphate buffer and diluted with the buffer so that the concentration of total solids was 0.5 per cent.

As shown in Table 2, the viscosity of all of the solutions was about the same and also showed no effect of flow rate. These results indicate that the preparatory procedures produced little or no degradation. However, any degradations which may have occurred during the extraction periods were not determined.

TABLE 2. COMPARISON OF THE VISCOSITIES OF MUCIN A WITH THOSE OF EXTRACTS AT 30.0°C

0.5% solutions of	Cannon-Manning viscometer			
	100		200	
	Centi-poise	η_r	Centi-poise	η_r
A	2.38	2.82	2.36	2.78
S ₁	2.23	2.64	2.20	2.59
S ₂	2.37	2.81	2.46	2.90

Effect of alkali on the viscosity of submaxillary mucin. Since the mucin was dissolved in alkali as part of its preparation and since some electrophoretic measurements were made at pH 10, the effect of alkali on the viscosity of mucin C was studied.

A 1% solution of mucin C (5 ml), dialysed at 2-4°C against 1 l. of a phosphate buffer solution of pH 7.4 and ionic strength 0.1 for 48 hr, had a viscosity of 4.55 cP at 30.0°C. After measurement of the viscosity, the mucin solution was dialysed against phosphate buffer of pH 10 and ionic strength 0.1 for 48 hr. The viscosity at pH 10 was 4.1 cP. The mucin solution was again dialysed against the phosphate buffer of pH 7.4 and ionic strength 0.1 for 48 hr, and its viscosity was 4.4 cP. Thus, although the viscosity was appreciably lowered at pH 10, it was virtually restored by adjustment to pH 7.4 even after keeping the mucin at pH 10 for several days at 2-4°C.

Optical rotation and ultra-violet absorption

Mucin C and a 2 cm tube were used. The specific rotation was: $[\alpha]_D^{25} = -23.2^\circ$ (0.87 per cent in water).

The ultra-violet spectrum of mucin A for a 40 mg per cent aqueous solution was closely similar to that for albumin, but the maximum shifted a little toward a shorter wavelength (265 m μ).

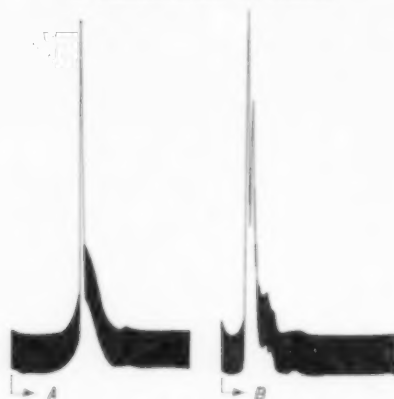


FIG. 2. Ascending electrophoretic patterns of mucin C (1% solution in a phosphate-NaCl buffer of ionic strength 0.1). (A) pH 7.8, 134 min, mobilities: -5.9 , -6.4 , -10.0 ; (B) pH 10, 70 min, mobilities: -7.4 , -8.3 , -10.7 , -11.7 , -15.8 ($\times 10^{-5}$ cm²/sec per V).

Electrophoretic mobilities of components of mucin at different pH values

The electrophoretic patterns of lyophilized submaxillary mucin C at pH 7.8 showed a principal peak merged with a smaller component and a small fast-moving peak (Fig. 2A). However, at least four components were evident at pH 10 (Fig. 2B). The change of mobility with pH is shown in Fig. 3. The mobilities given in Fig. 3 are the means of those from two or three measurements at the ascending boundaries for different periods of time during the electrophoresis run. As shown in Fig. 3, the mobilities of the components increased gradually with a rise of pH, and the components separated distinctly at pH 10.

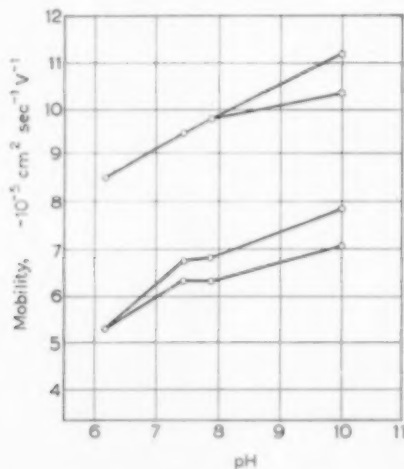


FIG. 3. Mobility vs. pH curves for mucin C (1% in a phosphate-NaCl buffer of ionic strength 0.1).

Ultracentrifugal components

As shown in Fig. 4 (A), two sharp and one diffuse peaks were present in the ultracentrifugal patterns of mucin C at pH 7.4. The sedimentation constants ($S_{20,w}$) of two sharp peaks were 2.5 and 2.1, respectively. However, a diffuse component which moved very fast (5.0 S) appeared more clearly at pH 10 (Fig. 4B), and the sedimentation constants of the two sharp peaks decreased somewhat to 2.4 and 1.95, respectively.

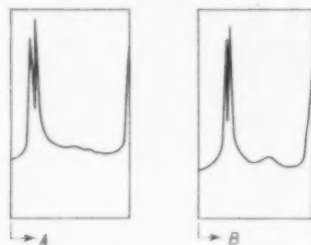


FIG. 4. Ultracentrifugal patterns of mucin C (2% in a phosphate-NaCl buffer of ionic strength 0.1 after 80 min). (A) pH 7.4, components: 2.5, 2.1 S; (B) pH 10, components: 5.0, 2.4, 1.95 S.

General chemical composition

The chemical analyses given in Table 3 show that the mucins prepared from the successive extracts of the cattle submaxillary gland were very similar in composition. They were composed of about 75 per cent protein and 25 per cent carbohydrate, of which the major portion of the latter was apparently hexosamine and sialic acid.

TABLE 3. CHEMICAL ANALYSES OF MUCINS A, B AND C

Constituent	% composition of mucin†		
	A	B	C
Total nitrogen (T.N.)	13.2	13.4	12.6
T.N. minus non-protein-N (Corr. T.N.)	12.0	12.2	11.4
Corr. T.N. \times 6.25 (Corr. T.P.)*	74.8	75.9	71.6
Biuret protein (as serum albumin)	70.4	70.4	58.8
Phenol reagent protein (as serum albumin)	73.9	75.9	70.0
Sialic acid (as O,N-diacetyl, DER-method)*	12.8	12.9	13.1
Carbohydrates (as glucose by anthrone)	1.1	1.3	1.1
Carbohydrates (as galactose by anthrone)*	2.1	2.4	2.2
6-deoxyhexose (cysteine-H ₂ SO ₄ method as fucose)	0.4	—	—
Hexuronic acid (as glucuronic)	0.3	0.3	0.3
Hexosamine (as N-acetylglucosamine)*	11.5	12.0	10.6
N-acetylgalactosamine	8.1	—	—
N-acetylglucosamine	3.4	—	—
Total (of items marked with *)	101.2	103.2	97.6

† After correction for ash (0.65 per cent as Na), moisture (6.3 per cent) and ether-soluble material (4 per cent).

A differential analysis of mucin A for galactosamine and glucosamine showed the major portion was galactosamine. The molecular ratio of galactosamine to sialic acid was unity (1.00:1.00).

The ratio of sialic acid to protein (phenol reagent) was closely similar for the three preparations (0.17, 0.17 and 0.19) despite the difference in the original extracts as shown in Fig. 1 (0.09–0.19).

The phenol reagent gave protein values which agreed fairly closely with those determined from the total nitrogen after correction for hexosamine and sialic acid nitrogen. The Biuret reagent, however, consistently gave lower amounts.

The content of carbohydrate as determined by the anthrone reagent was quite small and probably included the 6-deoxyhexose. The uronic acid content was very small.

Qualitatively, no phosphorus could be detected. Some of the samples showed, however, slight positive tests for ester sulphate.

DISCUSSION

The work described in this paper is a part of a broader study of the composition and nature of cattle submaxillary and sublingual mucins. Similar data for the sublingual mucin will be given separately (TSUIKI and PIGMAN, 1960). Further fractionations of these mucins usually yield products which no longer give typical mucin clots and will be described later.

The submaxillary mucin belongs to the class of mucoproteins as defined by MEYER (1953) and dissociates into several electrophoretic and ultracentrifugal components at alkalinities of pH 6 or greater. Some of the compounds responsible for the association appear to be strongly basic, since the maximum number of components is shown at pH 10. Since the viscosity was reversibly altered by the alkaline treatment, this dissociation did not seem to involve covalent bonds.

The acidic groups required for the associations to form mucin seem mostly derived from the sialic acid which is present to the extent of about 13 per cent. Although the extracts from which the three clots were made had different compositions, the clots had closely similar compositions, and in this respect are similar to those for synovial mucin (PIGMAN *et al.*, 1959) in which the acidic function is supplied by hyaluronic acid.

The total nitrogen (12.6–13.4) is a little higher than that given by HAMMARSTEN (1888) (12.3–12.4), BLIX (1936) (11.2–12.6) and TANABE (1939) (12.6–12.8). This difference may arise from the fact that the materials used in this work are the first clots obtained from the extracts and have received no reprecipitation, whereas the products prepared by the earlier workers were reprecipitated several times. BLIX (1940) reported the polysaccharide composition as 27 per cent, close to that found in this work. The principal hexosamine is galactosamine as indicated by isolation (BLIX *et al.*, 1952) and differential analysis by HEIMER and MEYER (1956) as was also done in the present work.

The source of TANABE's (1939) submaxillary mucin was not described but presumably came from the cattle glands. The optical rotation of his product and the

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hexosamine analysis agreed with those reported in the present work. His acetyl analyses (6.6–7.0 per cent) are a little higher than those calculated for the sialic acid and hexosamine determinations in Table 3 (5.4 per cent).

One of the most interesting features of the chemical composition is the equal molecular ratios of galactosamine and sialic acid. The data given by HEIMER and MEYER (1956) and ODIN (1958) also show a similar ratio for a product derived from cattle submaxillary mucin. The carbohydrate component, thus, seems to be composed of equal amounts of sialic acid and N-acetyl-D-galactosamine. The sialic acid seems to be the O,N-diacetylneuraminic acid (HEIMER and MEYER, 1956; BLIX *et al.*, 1956).

BLIX *et al.* (1952) indicated that the mucin contains small amounts of a "dihexose-hexosamine" polysaccharide (Kohlenhydrate II). Such a material would account for the glucosamine and hexose components found in this work, although the ratios are more for a "hexose-hexosamine" material. However, the amounts are small and close to the experimental error. In addition, the factors for glucose, galactose and fucose in the anthrone reaction are markedly different, but the hexose is probably galactose and or mannose (GOTTSCHALK, 1957; ODIN, 1958). Although the uronic acid content is very low, its presence seems definite since fractionation leads to fractions with larger amounts as will be described in later papers.

BLIX (1940) reported two electrophoretic components, one of which would currently be described as probably a salt boundary. The major "fast moving" component does not seem to correspond to those described in the present work, although the conditions were similar. The major component in the present work had an average mobility of -6.3×10^{-5} cm²/sec per V, at pH 7.4 in phosphate buffer at ionic strength 0.1. As will be shown later, the amount of the component corresponding to this mobility increased with further fractionation as the polysaccharide portion increased.

The submaxillary mucin appears to be a mucoprotein (coacervate) of protein cations and a glycoprotein anion which dissociates into two to four components over the pH range 6 to 10. The minor, rapidly migrating component with a mobility of -15.8 may not be necessary parts of the mucin and may be related to the small amount of sulphate often found.

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SHORT COMMUNICATION

THE CULTIVATION OF *TREPONEMA MICRODENTIIUM* AS SURFACE COLONIES*

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ORAL treponemes have been grown only rarely as surface colonies on solidified media (BERGER, 1958), and attempts to subculture these forms from surface growth have failed, with only two exceptions (FORTNER, 1929; BERGER, 1958). We have succeeded regularly in obtaining growth of surface colonies of these organisms in pure culture. We have also transferred single colonies through several successive passages and have isolated oral treponemes from mixed cultures as surface colonies. Success appears to have depended on the exclusion of atmospheric oxygen during manipulation of inocula.

All cultures were handled in a sealed chamber (Kewaunee C.B.R. Laboratory). Air inside the chamber was forced out by inflation of a twelve-foot meteorological balloon, and subsequently replaced by introduction of nitrogen into the chamber. Residual oxygen was absorbed by alkaline pyrogallol. The chamber atmosphere was circulated through a polarographic cell in a closed system during experiments and the oxygen concentration was recorded. All experiments were conducted at oxygen concentrations of 0.05 per cent or less. Media and materials were introduced into the chamber through an exchange lock.

The medium consisted of a veal heart infusion base with 10 per cent rabbit blood or horse blood and 3 per cent or 1.5 per cent agar. The medium was prepared 24 hr before being used and was stored in Brewer jars in 95 per cent H₂ and 5 per cent CO₂.

Stock cultures of *T. microdentium* in ascitic fluid broth were streaked on the rabbit blood 3 per cent agar medium in the nitrogen chamber and sealed in Brewer jars. The jars were evacuated and filled with 95 per cent H₂ and 5 per cent CO₂ and incubated for 4 days or more at 37° C. All of seven strains tested grew well as surface colonies in each of four such experiments, a total of twenty-eight trials (see Figs. 1, 2). Heavily inoculated "bench-streaked" controls exhibited light or questionable growth, unsuitable for transfer, in five of ten trials. Single colonies, from four of five strains, inoculated in the chamber were transferred successfully (in the chamber) to fresh plates. Single colonies were also transferred successfully to a semi-fluid medium (0.3 per cent agar) in twenty-one of twenty-four trials.

* This project was supported in part by a Research Grant, D-579, from the National Institute of Dental Research.

Another experiment employed as inocula seventeen samples of human gingival microflora grown anaerobically in spirochaete cup plates. Horse blood 1.5 per cent agar plates were streaked in the nitrogen chamber and sealed in Brewer jars. Controls consisted of medium streaked outside the chamber and transferred to Brewer jars. All jars were evacuated and filled with 95 per cent H_2 and 5 per cent CO_2 and incubated as above. Isolated colonies of *T. microdentium* appeared on or in the agar in large numbers from each of the seventeen samples. No spirochaetes grew on the "bench-streaked" controls.

These experiments suggest a high sensitivity to oxygen as an explanation for previous failure to obtain isolated surface colonies of indigenous spirochaetes of man. The method will facilitate the isolation and cultivation of pure cultures for systematic study of these organisms.

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CULTIVATION OF *TREPONEMA MICRODENTITUM* AS SURFACE COLONIES

T. microdentitum colonies on rabbit blood agar after 7 days.

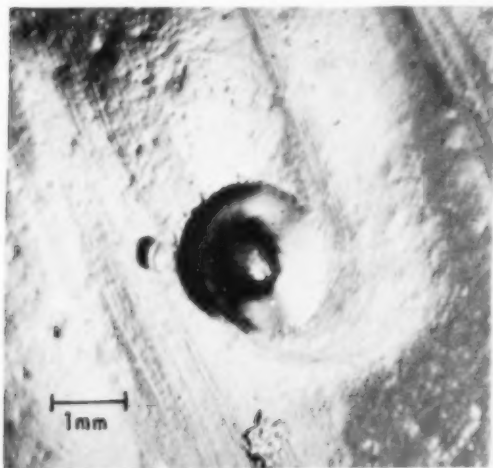


FIG. 1. Smooth, umbonate, 2 mm colony.

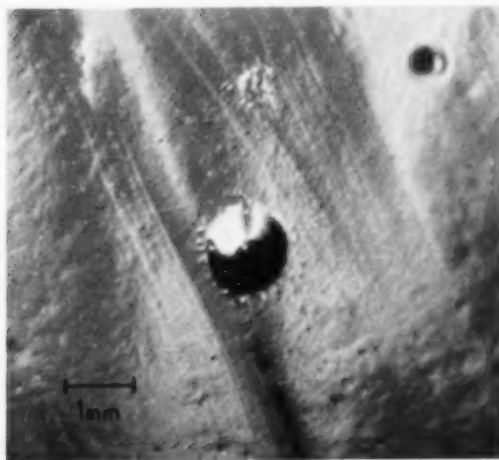


FIG. 2. Convex, 1 mm colony.

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A STUDY OF ORGANISMS ASSOCIATED WITH INJECTIONS INTO THE ORAL MUCOSA

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Abstract—Bacteria were demonstrated histologically in needle tracks produced in freshly excised human gingiva. Organisms of various types were cultured from the solutions ejected from hypodermic needles after intra-oral injections where there was no prior treatment of the mucosa but no organisms were cultured if the mucosa was dried beforehand. Culturing from mucosa also showed that drying it by wiping with gauze removed most of the bacteria.

THE topical application of antiseptics to the oral mucosa prior to an injection is postulated as a routine procedure by the great majority of current textbooks of dental surgery. When topical application of antiseptics is mentioned in the literature, it is invariably recommended (KANTOROWICZ, 1929; WIDDOWSON, 1950; MEAD, 1951; THOMA, 1952; FISCHER, 1955).

The contrary view, that antiseptic measures are not required as a preliminary to intra-oral injections, cannot be found in the literature, but there is abundant reason to suppose that the practice of injecting without preliminary antiseptic treatment of the mucous membrane is indeed common. Thus, a recent survey in the United States revealed that out of 666 dental practitioners 47.6 per cent did not, as a matter of routine, use topical antiseptics prior to local intra-oral injections. An additional 1.7 per cent stated that they used these agents occasionally (ZINNER and STREITFELD, 1958). This may be considered a conservative estimate, as some practitioners might well have been reluctant to admit the omission of this procedure.

Despite these findings, the literature reveals few, if any, cases where the omission of topical antiseptics has been conclusively shown to be the direct cause of an infective process. It appeared desirable, therefore, to find out whether bacteria could, in fact, be carried from the surface of the mucous membrane of the mouth into the deeper tissues during an intra-oral injection, and for this purpose the experiments reported below were carried out.

The presence of bacteria in the needle track

A search of the literature has not revealed any reference to the presence of bacteria in the needle track, but as MÜLLER (1950) has shown that epithelium may be carried into the submucosa by the needle of a hypodermic syringe, it seems reasonable to suppose that micro-organisms might readily be introduced into the deeper tissues in the same way.

In order to test this hypothesis, experimentally "injected" tissues were examined for the presence of bacteria. A sterile 26 gauge needle was introduced into a freshly excised portion of human gingiva in exactly the same manner as if an injection were being made. The tissue was then fixed immediately in 10 per cent formol-saline. The mucous membrane was not subjected to preliminary treatment and the presence of the normal flora was assumed. Material from five patients was examined, serial sections of the gingiva being prepared and alternate sections stained with haematoxylin and eosin, and with Gram's stain. Three of the five specimens examined showed the presence of bacteria in the needle track. The micro-organisms could be seen in the sections stained with haematoxylin and eosin but were more easily identified in the sections stained by Gram's method (Figs. 1 and 2).

Isolation of bacteria from the needle following an injection

In order to find out whether organisms can be isolated from a hypodermic needle immediately after use, the following experiment was performed.

A sterile dental syringe charged with a cartridge of 2% Xylotox solution (adrenaline 1:80,000) was used for a routine intra-oral injection in living patients without any preliminary treatment of the oral mucosa. After withdrawal, the first two drops in the syringe needle were discharged into 10 ml of glucose broth, which was then divided into two portions, for aerobic and anaerobic cultivation, and incubated for 24 hr. Subcultures were then made on blood agar and incubated for a further 24 hr. Table 1 shows the organisms isolated from the needle in the eighteen cases examined.

TABLE 1. ORGANISMS ON THE SYRINGE NEEDLE
(number of cases = 18)

	Cases
<i>Streptococcus viridans</i>	16
<i>Neisseria catarrhalis</i>	4
<i>Micrococcus tetragenus</i>	2
<i>Staphylococcus pyogenes</i>	1
<i>Staphylococcus albus</i>	1
Diphtheroids	1
β -Haemolytic streptococcus	1
Non-haemolytic streptococcus	1

The effect of drying the oral mucosa upon the local bacterial flora

The efficiency of various chemical agents recommended as intra-oral antiseptics has been tested by various authors, amongst them McCULLOCH (1945), RUBBO and PIERSON (1952) and MILLER and APPLETON (1931) who have examined these agents in great detail. Since drying the mucosa is usually recommended prior to the use of such antiseptics, it was decided to investigate the effect on the local bacterial flora of this procedure alone, without the subsequent application of an antiseptic. Two series of experiments were carried out.

In one series the buccal sulcus of the lower right premolar region was isolated with sterile gauze and a swab was plated out on 5 per cent blood agar to demonstrate the presence of the resident flora. The mucosa was then dried by rubbing the area several times with sterile gauze, and a second swab was then taken and cultured in the same manner. The swabs were previously moistened in sterile glucose broth.

The 110 cases used for this series of tests showed an abundance of micro-organisms on the moist mucosa, *Streptococcus viridans* predominating. When the mucosa was dried with sterile gauze most of the culturable resident flora was removed, and amongst the few organisms remaining *Streptococcus viridans* again predominated (Table 2).

TABLE 2. ORGANISMS ISOLATED FROM THE MUCOUS MEMBRANE BEFORE AND AFTER DRYING WITH STERILE GAUZE (number of cases = 110)

	Before drying	After drying
<i>Streptococcus viridans</i>	101	82
<i>Staphylococcus albus</i>	46	13
<i>Neisseria catarrhalis</i>	64	32
β -Haemolytic streptococcus	15	6
<i>Staphylococcus pyogenes</i>	5	2
<i>Streptococcus pneumoniae</i>	1	0

In the second series of experiments a sterile dental syringe charged with a new cartridge of 2% Xylotox (adrenaline 1:80,000) was used for an injection in living patients in roughly the same area as above, after careful and thorough drying of the mucosa with sterile gauze. After withdrawal, the first two drops of the solution in the syringe needle were discharged into glucose broth. This was incubated aerobically and anaerobically for 18 hr and subcultured on to blood agar.

For this series, patients from the first experiment were used; also the figures indicating the incidence of micro-organisms in the syringe before drying, were taken from Table 1.

Table 3 shows that after drying the oral mucosa no organisms were isolated from the two drops in the lumen of the syringe in each of the eighteen cases examined.

TABLE 3. ORGANISMS ISOLATED FROM THE SYRINGE NEEDLE BEFORE AND AFTER DRYING THE MUCOUS MEMBRANE

	<i>Strep. viridans</i>	<i>Staph. albus</i>	<i>Neiss. catarrhalis</i>	<i>Micrococ. tetragenus</i>
Before drying (from Table 1)	16	1	4	2
After drying	0	0	0	0

DISCUSSION

The experiments described above show that organisms can regularly be isolated from the needle following an intra-oral injection but, when the mucosa is dried as a preliminary measure, the incidence of isolation is greatly reduced. Drying alone does not, of course, sterilize the mucosa; the fact that no organisms were isolated from the needles after drying is not in any way conclusive, since in this study only organisms readily cultured were recorded.

The present findings support those of MÜLLER and indicate that some sterilizing agent prior to an intra-oral injection appears advisable, since the potentialities for infection exist. FISCHER (1955), referring to the medico-legal aspect of local injections without prior antiseptic treatment of the mucosa, points out that the operator may be held responsible and, in fact, may be accused of negligence in a case of infection due to an injection.

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FIG. 1. Section of human gingiva showing a needle track. Haematoxylin and eosin. $\times 100$.

FIG. 2. Higher magnification showing micro-organisms in the needle track. Gram's stain. $\times 1000$.

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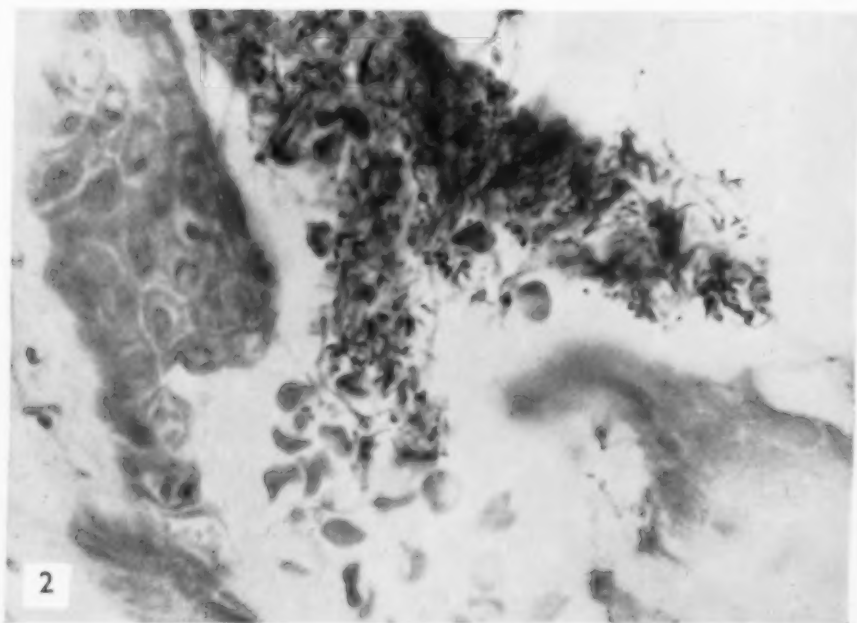
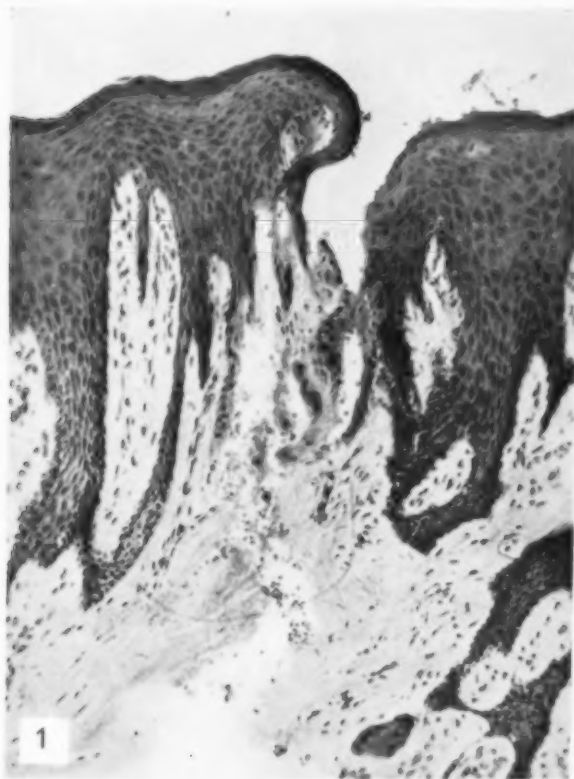


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GINGIVAL BIOPSY AS A DIAGNOSTIC AID IN AMYLOID DISEASE*

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Abstract—The result of gingival biopsy was evaluated in ten patients with known amyloidosis. It was positive in six, five of whom had no associated disease. The crystal violet stain provided the most reliable histologic evidence of the presence of amyloid. The gingival biopsy was positive in five patients with amyloidosis in whom there were no clinically obvious changes in the mucous membranes of the mouth. There was no consistent correlation between the results of the gingival biopsy and the Congo red test. It would appear that these tests supplement one another in establishing the diagnosis of amyloid disease.

AMYLOIDOSIS is a disease which is unknown in its pathogenesis, uncertain in its nature, and difficult in its therapy; it is, moreover, all too frequently missed in diagnosis. Of the three cases of primary amyloidosis discovered at autopsy at the Massachusetts General Hospital in a five-year period (1950-1954), none was diagnosed prior to death.

Difficulty in the diagnosis of amyloidosis stems to a large extent from the many ways in which it may present (RUKAVINA *et al.* 1956), such as polyneuropathy, nephrosis, or isolated tumour. There is no single clear-cut syndrome of amyloidosis. Further difficulties are encountered in substantiating its presence. The Congo red test, the most commonly used tool, provides evidence in a number of cases in which the diagnosis of amyloidosis is suspected (STEMMERMAN and AUERBACH, 1944). In certain disease states such as rheumatoid arthritis many of the patients with long-standing disease have borderline Congo red values (50-70 per cent retention in the serum) (DIXON, RAMCHARAN and ROPES, 1955). On the other hand, in some cases of so-called primary amyloidosis unassociated with chronic inflammatory disease, the test may be negative (SYMMERS, 1956; MATHEWS, 1954). It is not known whether this is due to peculiar dye-binding characteristics of the amyloid or to a smaller total mass of amyloid.

Tissue biopsy, first reported in 1925 and 1928 (RUKAVINA *et al.* 1956), is the only present method of establishing a definitive diagnosis of amyloidosis. In selected cases, liver, spleen, kidney and skin have all been biopsied (SYMMERS, 1956; MATHEWS, 1954; GOLDEN, 1945; RIGDON and NOBLIN, 1949). Many of these techniques involve

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problems of the limited accessibility of the tissues, the potential hazard of haemorrhage and sepsis, or the fact that the amyloid may be deposited elsewhere.

The gingival biopsy was studied extensively by SELIKOFF and co-workers (SELIKOFF and ROBITZEK, 1947; SELIKOFF and HERSCHFUSS, 1949), GORLIN and GOTTSCHEN (1949), MEYER (1950) and LIGHTERMAN (1951) in groups of tuberculous patients who had a high incidence of amyloidosis. The results were encouraging, but the technique has not been generally used except for isolated studies (POCOCK and DICKENS, 1953; ANDRADE, 1952). Indeed, in primary amyloidosis, several authors state that the procedure yields so few positive results that it is of little value (SYMMERS, 1956; COOKE, 1958).

The present study was undertaken to assess the value of the gingival biopsy in cases of proven amyloidosis of various types. The use of special stains and optical techniques to aid in the identification of amyloid is described. In this series, the biopsy was positive in five of eight cases of primary amyloidosis, and one of two cases with proven secondary amyloidosis.

TECHNIQUES

A group of ten patients with proven amyloidosis was selected for gingival biopsy. Amyloid was known to be present in these patients by previous tissue biopsy and in some was confirmed on subsequent post-mortem examination (Table 1). As controls, fifty patients with rheumatoid arthritis, but no clinical evidence of amyloid, also were biopsied.

After infiltration of 2% xylocaine hydrochloride into the mucobuccal fold in the canine fossa, a 2-3 mm strip of mucoperiosteum, extending from the labial sulcus to the crest of the dental alveolus in the maxillary canine-premolar areas, was excised. Bleeding was well controlled with simple gauze sponge pressure, and no sutures were required. The patient's dentures, when available, were replaced immediately after biopsy to serve as a pressure dressing. Healing progressed satisfactorily, without secondary sepsis or protracted discomfort. The tissue was fixed in 10% formol-saline solution and later stained with haematoxylin and eosin, Congo red, crystal violet and van Gieson stains (COHEN, CALKINS and LEVENE, 1959). These sections were examined with the light microscope and with a Reichert fluorescent microscope at 365 m μ .

RESULTS

(a) *Oral examination.* This revealed five edentulous and five non-edentulous patients. Only one patient, with advanced skin and mucous membrane lesions, showed extensive multiple papillary projections resembling multiple raised angiomas and an enlarged, waxy, thickened, nodular tongue (Fig. 1). None of the nine other patients showed any alteration of oral mucosa which would suggest amyloid deposits. With the single exception noted, none of the tongues examined were pathologically enlarged or otherwise abnormal, despite the presence of amyloid confirmed on post-mortem examination. The edentulous patients showed physiological enlargement of their tongues characteristic of edentulous mouths, without loss of lingual papillae.

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TABLE 1. GINGIVAL BIOPSY IN PROVEN AMYLOIDOSIS

Case No.	Hospital No.	Sex	Age	Results of biopsy	Comment on histology	Congo red test*	Associated disease	Other tissues involved†	Type of amyloid
(1) G.P.	M.G.H. 345663	M	55	+	Almost complete replacement of corium with amyloid. Thin atrophic epithelium	Not done	None	Skin, tongue, blood-vessels, heart, spleen (P.M.)	Primary systemic
(2) A.D.	Mt. Auburn	M	63	+	Minute perivascular deposit seen only after repeated sectioning	61%	None	Lungs, heart, blood-vessels, kidney, spleen (P.M.)	Primary systemic
(3) ‡E.D.	M.G.H. 1017053	F	53	+	Papillary area of corium plus perivascular involvement	1%	None	Liver, kidney, spleen, adrenal, heart, blood-vessels, tongue (P.M.)	Primary systemic
(4) ††J.P.	M.G.H. 1035784	M	38	+	Largely perivascular but some nodular accumulation	60%	None	Blood-vessels, nerve, skin, muscle (B)	Primary systemic
(5) P.C.	M.G.H. 139712	M	51	+	Small perivascular accumulation	36%	Chronic granulomata of unknown aetiology	Renal glomeruli (B)	Secondary
(6) M.C.C.	Middlesex Sanatorium 7779	F	42	+	Perivascular and nodular accumulation	12%	None	Liver, lymph node, bone marrow (B)	Primary (? systemic)
(7) M.F.	M.G.H. 986663	M	50	—	—	0%	Nephrotic syndrome	Renal glomeruli (B)	Primary (? systemic)
(8) **M.B.	M.G.H. 19603	F	48	—	—	16%	Chronic pyelonephritis, osteomyelitis	Kidney, spleen, liver, thyroid, pancreas, small intestine, adrenal, bone marrow, blood-vessels (P.M.)	Secondary
(9) M.S.	M.G.H. 1006130	F	54	—	—	70%	None	Laryngeal tumour (B)	Primary localized
(10) S.S.	M.G.H. 988923	M	66	—	—	33%	Nephrotic syndrome	Renal glomeruli (B)	Primary (? systemic)

* Percentage retained in serum after 1 hr.

† P.M. = post-mortem examination.

B = biopsy.

‡ Clinico-pathological Conference No. 44461, *New Eng. J. Med.* 259, 979, 1958.** Clinico-pathological Conference No. 44281, *New Eng. J. Med.* 259, 88, 1958.

†† Also studied at the Johns Hopkins Hospital, Case No. 730801.

Of these ten patients, there were six males and four females. The ages ranged from 38 to 66 years of age (Table 1). The gingival biopsy was positive in six patients (four edentulous and two non-edentulous). It was negative in all fifty control patients.

(b) *Histology.* Of the various staining techniques used, the appearance of metachromasia with crystal violet was the most useful, and clearly demonstrated amyloid

in the six positive biopsies. The haematoxylin and eosin stain, though necessary for orientation, did not materially contribute to the diagnosis and, indeed, if it were not supplemented by the other stains, the diagnosis often would have been missed (Figs. 2, 3). In only one case was there a significant amount of extra-cellular hyaline material suggestive of amyloid observed with this stain (Fig. 4). The Congo red stain was usually faintly positive in the suspected areas, but was of little differential value since the dense connective tissue in the corium of the gingival mucosa also appeared positive. The van Gieson dye (staining collagen red and amyloid yellow-orange colour) was helpful in ruling out the false positives found by Congo red staining. Fluorescent microscopy of Congo red stained sections, though of some value in studying organs in which adequate decolorization is possible (COHEN, CALKINS and LEVENE, 1959) was not as useful, because of the densely packed collagen fibres. It is interesting to note that whether or not a given patient had a positive Congo red test, the amyloid, when viewed histologically, invariably exhibited some affinity for the Congo red stain.

Where present, the amyloid was perivascular in location, though small nodular deposits without any clear-cut relationship to blood-vessels were also found (Fig 3). In Case No. 1 (G.P.) there was almost complete replacement of the corium with amyloid (Fig. 4). The deposits were as often seen in the papillary layers as in the deeper dermal tissue.

(c) *Congo red retention.* Congo red tests (except for Case No. 2, A.D.) were performed by the acetone precipitation method in the laboratory of Dr. M. W. ROPES. There was no consistent correlation between results of these tests and the presence or absence of amyloid in the gingival specimen. Of the six patients with positive gingival biopsies, one patient had a retention in the serum of 1%, a second of 12%, but in three others the retention was 36%, 60% and 61%. Thus, in only two of these were the results of the test definitely positive. The Congo red test was avoided in the sixth patient with massive amyloid infiltration of the skin and mucous membranes.

The Congo red tests in those patients with negative gingival biopsies were 0 per cent in one patient, revealed 16 per cent retention in a second, and 33 per cent and 70 per cent in the final two patients.

DISCUSSION

The above cases clearly illustrate the value of the gingival biopsy in demonstrating the presence of amyloidosis in some individuals. Of the total of ten cases, the gingival biopsy was positive in six. In five of these, the disease was not accompanied or preceded by any other known inflammation or disorder. One patient exhibited a diffuse granulomatous involvement of the lymphoid tissue, the nature of which has not yet been defined. Three other patients with the so-called primary form of the disease, and one patient with amyloidosis secondary to chronic infection, had negative tests.

There was no consistent clinical difference between the patients who exhibited positive gingival biopsies and those in whom the biopsy was negative. Bone marrow

aspiration, paper strip electrophoresis (for "M" spot) and urinalysis for Bence Jones protein were carried out in all instances. In none was evidence of multiple myeloma found (TILLMAN, 1957; CAHN, 1957; BRUNSTING and MACDONALD, 1947). In some patients with disease primarily localized to the kidney, the biopsy was positive. In one with widespread perivascular involvement, the gingival biopsy was negative.

Advantages of the gingival biopsy are to be found in the relative simplicity of the technique which requires a minimum of preparation, assistance and equipment. A small amount of tissue may be removed with little or no postoperative discomfort to the patient. The presence or absence of teeth does not appear to alter the gingival deposition of amyloid. Finally, and most important, is the fact that a positive gingival biopsy may be expected in a significant number of cases, even when there is no clinically obvious change in the mucous membranes of the mouth.

In our opinion, all patients suspected of having amyloidosis should be subjected to a gingival biopsy and a Congo red test. The former procedure is completely benign and the latter is entirely safe, in our hands, provided that recrystallized material is used. Together, these tests provide a reasonable likelihood of establishing the diagnosis in patients with amyloid disease.

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FIG. 1. Tongue of patient No. 1 (G.P.), demonstrating macroglossia, and multiple raised, irregular and nodular, waxy deposits of amyloid.

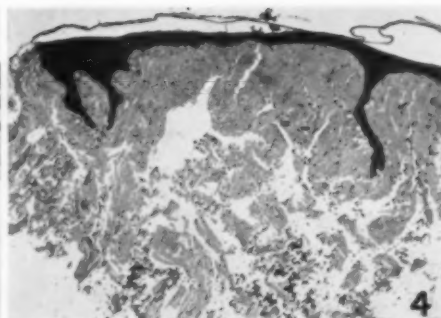
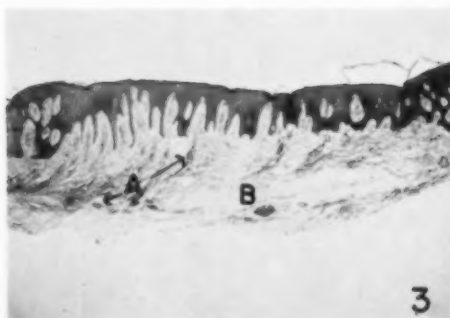
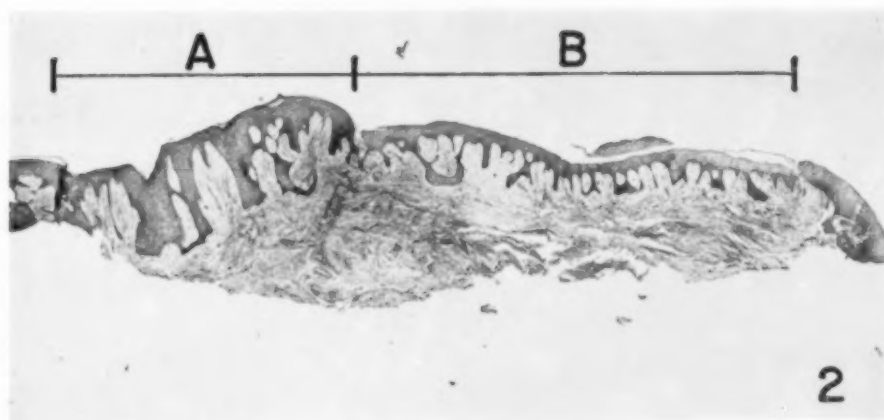
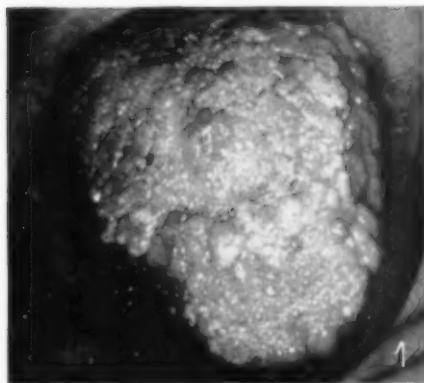
FIG. 2. Biopsy site—normal gingiva. At A is normal epithelium, a keratinized squamous zone, present at the alveolar-dental area. Note change to a lower, non-keratinized, squamous epithelium in the region of the unattached areolar gingiva at B.

Papillary projections of the corium are a feature of the alveolar-dental zone and the epithelial rete pegs are reduced in the areolar gingiva of the muco-buccal fold (and in edentulous gingiva). Haematoxylin and eosin. $\times 30$.

FIG. 3. Gingival biopsy—patient No. 3. Metachromasia (red) characteristic of amyloid is seen about blood-vessel (A) and in an isolated nodule (B) in the corium, which appeared normal with haematoxylin and eosin. Crystal violet. $\times 21$.

FIG. 4. Gingival biopsy—patient No. 1 (G.P.). Patient edentulous. Note thinning of epithelium and loss of rete pegs. Corium is replaced by massive amounts of amorphous, glassy, eosinophilic substance. Corium and deeper layers were friable and bled easily. Haematoxylin and eosin. $\times 21$.

GINGIVAL BIOPSY AS A DIAGNOSTIC AID IN AMYLOID DISEASE



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A SURVEY OF THE ORAL HEALTH OF NAVAJO INDIAN CHILDREN

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Abstract—A survey of the oral conditions of 1347 Navajo Indian children between the age of 3 and 20 years was conducted.

The data recorded were, the gingival condition about each tooth, deposits, abrasion and dental caries. The prevalence, severity and incidence rate of gingivitis were found to be remarkably similar to that of a group of Alabama school children. The main differences were a high incidence of subgingival calculus at an early age, a high incidence of abrasion, a different intra-oral pattern of gingival disease and a low caries rate.

The diet was studied of the Navajo Indians residing in the remote areas which were visited during a family to family survey.

The mode of life, general health, climatic conditions are discussed with possible reference to the oral health findings.

INTRODUCTION

THE object of the survey was to examine the teeth and oral mucosa of people living under conditions and subsisting on a diet different from those we consider conform to modern living standards.

The Navajo Indians keep to a traditional dress, mode of life and language. They live in a sandy arid district, three-quarters of which is classed as desert, and their semi-nomadic existence requires that they live simply and have few possessions. Their diet is limited to what can be produced or bought from the trading post. Agriculture is restricted and, even in areas where water is available, only certain crops will grow.

The gingival condition of these people might well be affected in one or more of several ways. Dietary deficiencies would be expected as there is an almost complete lack of fresh fruits and vegetables; the chewing of tough foods stimulates the gingival tissues and prevents debris from collecting about the teeth, and alteration of the shape of the teeth by abrasion from sand, which is inevitably taken into the mouth with food, alters occlusion and rarely allows cuspal interference to develop.

THE PEOPLE AND COUNTRY

The Navajo is the largest single Indian tribe in the United States and is increasing at the rate of 2.5 per cent per annum; in 1868 there were approximately 9000 Navajos and in 1958 the number was estimated to be over 85,000 (YOUNG, 1957). The resources of the Reservation are meagre and can hardly support 35,000 people. Many families find employment elsewhere either in a seasonal capacity or under a re-allocation programme, which must continually increase in extent, but the majority

of the tribe live on the Reservation and many families, particularly those in the more remote areas, adhere to their traditional way of life as sheep herders.

The Navajo Indian Reservation is 23,500 square miles in extent and occupies parts of Arizona, New Mexico and Southern Utah. Although the natural resources necessary for subsistence are scattered, few areas are without inhabitants, and the outstanding feature of the Navajo settlement is the way in which the people are dispersed over this vast area. There are no communities large enough to be classed as villages except those which have come into existence around the few government schools, hospitals or administrative centres.

Navajo families are not nomadic in the sense of a continual wandering, but they lead an apparently semi-nomadic existence due to the necessity of finding pasture for their sheep and goats. In winter they return to their hogans and, during the planting season, remain near their fields.

There are four types of country—alluvial valleys, upland plains at 6000 ft, mesas at 7000 ft, and mountains up to 10,000 ft. Each of these is cut by deep canyons. The humid areas are limited to the mountains which form only a small part of the Reservation. The steppe zone immediately below the humid zone has 12–16 in. of rainfall but comprises only one-fifth of the area, while the remainder is classed as desert having a low rainfall which occurs as summer downpours of high intensity and short duration. These desert and highland plains provide sparse grazing. The high mesas are covered with sagebrush, pinon and juniper, but grassland meadows are found only in the most humid mountain areas and are accessible only part of the year for they may be snowbound from December to April.

Such country does not support a large number of people or animals. Flowing water is rare and the low rainfall allows the soil to dry out to some depth. In addition, extremes of temperature, a high evaporation rate, dust storms and overgrazing for many years has resulted in soil erosion.

Much of Navajo family life revolves round the grazing of their herds of sheep, goats and horses. The family may move several times during the year but usually within well defined areas. In these areas they build hogans of timber and adobe or summer shades of poles supporting a roof of brushwood. The animals are grazed in the surrounding country and are brought back each night to a corral. The children play an important part in the herding of animals and walk many miles during the day. The term "family" is broad in Navajo society and often includes several biological units; for groups of up to twenty persons, but usually fewer, may live together.

Agriculture, although limited, is important in the Navajo economy. Almost every family raises some of its food and some families live for weeks on their own produce. In the areas besides rivers, irrigation produces reasonable crops and the steppe zone supports some growth. Crops include corn, melons and squash and, in some areas, beans, potatoes, turnips and other vegetables, but families usually raise only one type of produce and few make their living wholly by agriculture. In rare isolated areas orchards with apricots, grapes and apples, together with melons and other ground crops, provide luxuries for the few but contribute insignificantly to the feeding of the tribe.

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A considerable amount of food is obtained from the trading post which is also the means of entry for all manufactured goods. The trading post functions as an old-time rural general store where everything can be purchased and the storekeeper acts as banker, pawnbroker, merchant and post office official. Although there are now some wage earners amongst the Navajo population, groceries and other goods are mostly purchased by trading sheepskins, woven rugs and blankets. Capital is invested in silver and turquoise jewellery which is pawned or sold when necessary.

Oil and uranium have been found on the Reservation during the last 10 years. The proceeds from these go, not to individuals, but to the Tribal Council which has built schools and roads and otherwise improved the Reservation. A general increase in the standard of living is taking place and the pick-up truck is superseding the horse as transportation.

The first schools were opened in the 1870s but although in 1947 11,000 children were attending school, some 15,000 were unable to do so. However, in 1954, due to the extensive building programme and an increasing awareness of the need for education amongst the Navajos themselves, only 9 per cent of the children were not attending school and many of these lived in remote areas.

With education and the increasing opportunities, the life and habits of the Navajo people are changing, a trend which is even mirrored in their dental health. Some of the Navajo population now earn wages and as wage earners are often employed close to food markets, thus their diet tends to be different from those who still live in remote districts. A part of this survey was, therefore, carried out in as remote an area as could be found.

THE DIET

The main articles of diet in the remote parts of the Reservation are mutton or goat, bread made from wheat flour bought at the trading post, coffee, sugar and shortening.

Three methods of making bread were seen. The dough, consisting of white flour, baking powder and water, is kneaded into flat cakes; these are cooked, either over an open fire on a tin plate with a little shortening or in deep fat, and resemble tortillas rather than a loaf of bread.

Meat is mainly mutton and almost the entire animal is eaten, which probably offsets the lack of fresh vegetables. Some of the meat is dried in thin strips and stored. The remainder is stewed, baked or cooked over an open fire and, while it lasts, is eaten at every meal. The family eats together at dawn and dusk but no regular meal is taken at midday.

The children are weaned at the age of 10 months to 1 year and go directly on to the adult diet of meat, bread and coffee. Although supplemental breast milk may be given for a year or longer, cows' or goats' milk is rarely used.

Some articles of diet are dependent on region and season. Corn, pumpkin, squash, beans, potatoes and canned foods form an occasional dietary addition. Breads, sweet rolls, pies, cooked sweets, ice creams and soft drinks are available at some of the trading posts, but these are unusual luxuries rather than articles of diet. On rare

occasions fresh fruit is purchased in season, often regardless of price, and this is eaten on the shopping trip or immediately on return home. Consumption of wild fruits and berries has been practically abandoned, but children were observed collecting squaw berries which are dried and ground and made into a preserve. Pinon nuts are eaten when available.

Water is scarce in all areas of the Reservation and although a few families live near a river or irrigate from a spring or well, most have to carry water for many miles. Water is carried in all types of containers and kept with considerable care but contamination occasionally takes place, as would be expected.

GENERAL HEALTH

The most prevalent diseases amongst the Navajo are the common infections: pneumonia, gastritis, enteritis, measles and tuberculosis (HADLEY, 1955). These diseases are also the commonest cause of death, particularly in infancy. Heart disease, vascular lesions of the central nervous system and malignant neoplasms, which are the leading causes of death in the United States, are not common in the Navajo. In 1955 the death rate of the Navajo was 9.8, and for the rest of the United States 9.6 per 1000 population, but the Navajo dies at an earlier age; for instance in infancy the death rate was 4.9 times higher in the Navajo, and 60 per cent of deaths occurred below the age of 15 years compared with 9 per cent for the rest of the United States (YOUNG, 1957).

The popular press has attributed the high incidence of disease to dietary deficiencies but this is without scientific support. Nutritional diseases amongst 60,000 patients were reported as pellagra 10, scurvy 2, beri-beri 2, rickets 1 and malnutrition 97 (DARBY, 1956). They found that the supply of vitamin C in the diet was restricted and the ascorbic acid levels of the serum were low, at least half the children of all groups having less than 0.3 mg per cent, but there was no correlation between the presence of gingival lesions and the serum levels of ascorbic acid. In one area, however, where the lowest serum levels were found the prevalence of gingival disease was greatest.

In the present survey all families questioned had a very low intake of fresh fruit and vegetables except two families who had small orchards at the bottom of a canyon beside a spring. The supply of vitamin C in the diet is probably derived from fresh meat for, although the vitamin C content of muscle is low, the content of liver and other organs is high and the entire animal is consumed.

CHILDREN EXAMINED IN THE SURVEY

The Navajo Mountain area south of the San Juan River is one of the most inaccessible and unaltered parts of the Reservation and was the one chosen for a family to family study. Here, 53 children between the ages of 3 and 20 years were seen. In addition, the areas of Bluff, Montezuma Creek, Hatch and Aneth provided 148 children. Roads in these areas are hardly more than rough tracks and were traversed by a four-wheel-drive jeep and, in places, on foot.

The semi-nomadic habits of the sheep farmers and their families made them difficult to find and as these isolated areas are wild, rough country cut by deep

canyons and sparsely inhabited, many long and fruitless journeys were made. Consequently, the number of children examined in these areas was small and in order to examine greater numbers an additional 1146 Navajo children between the ages of 11 and 20 years from all parts of the Reservation were examined on their first day at boarding school. Approximately half of these children had not been to boarding school before, the remainder had lived in school for one or more years. As boarding school regime apparently affects gingival and dental health, they are listed separately.

As a basis for comparison, 122 children between the ages of 11-16 years attending Junior High School in Gardendale (a commuter community of Birmingham, Alabama) were examined by exactly the same technique. In addition, 395 children in the Deaf and Blind School in Alabama, who participated in an oral hygiene programme for 2 years, and 404 children between the age of 6 and 18 years attending high school in York (a rural community in Alabama) are included but, although the same technique of examination was used for these children, fewer data were recorded and they can be included in only some of the Tables.

METHOD OF EXAMINATION AND SCORING

Throughout this paper the term "prevalence" is used to mean the presence or absence of a condition; "incidence" means the extent to which a condition extends amongst a number of units at risk contained within an entity, such as the number of affected teeth in a mouth, and "severity" means a level or grade of intensity into which a condition may be subdivided.

All the children were examined by the same examiner. A separate chart was used for each child and the items recorded were: the gingival condition about each tooth, the type of deposit on each tooth, the degree of attrition, D.M.F. teeth, the depth of the crevice or pocket, and the occlusion. This report considers the first four items.

The criteria for the different levels of severity of gingivitis are (a) a zero level where no clinical evidence of inflammation is present or is so slight that it could be detected only by a research worker, (b) a one-plus grade where there is slight redness, swelling, and loss of stippling, a level generally recognized in clinical practice as a mild gingivitis, (c) a two-plus grade where symptoms are present, such as bleeding, tenderness or pain, and (d) a three-plus grade where there is gross swelling, redness, ulceration, and severe pain or spontaneous haemorrhage. The types of deposit recognized were calculus and soft deposit. Attrition of teeth was recorded as: no recognizable attrition, facets in the enamel, exposure of dentine, and gross loss of tooth structure. Dental caries was only recorded at the stage of obvious loss of tooth structure and where restorations had been inserted. The reason for including caries experience in this survey was that loss of contact point, marginal ridge, or occlusal surface, drifting or tilting of teeth due to destruction by caries and avoidance of the use of areas of the mouth due to pain, cause stagnation of food, irritation to the gingiva and localized areas of gingivitis; restorations often do not eliminate the gingival irritation. As no criteria were available to distinguish between carious areas and restorations which were or were not potentially harmful to the gingiva, all restorations were recorded and all diseased teeth where clinical loss of tooth surface

was present. Standardized explorers, as used in previous dental caries surveys, were not used (PARFITT, 1954). The present findings cannot be directly compared with dental caries surveys of other groups where incipient and early caries are recorded, unless cavitation with obvious loss of tooth structure is specifically recognized as a level of severity. The caries experience of groups within this survey, however, are comparable as the same technique was used throughout.

Field-survey conditions were difficult and, as the examination itself could not be hurried, the extent of the examination was limited to the buccal surfaces of the right side of the mouth for the gingival condition about each tooth, the type of deposit present and the depth of the pocket or crevice. This reduced the time by four and allowed the required number of children to be seen with little loss of data. The use of artificial light in much of the present survey was out of the question and as daylight is preferable in the assessment of colour it was used throughout the study. The children were examined away from direct sunlight facing an expanse of sky and in school were examined close to a large window.

The gingival score is compiled from the recorded clinical condition surrounding each tooth by giving a numerical value to each of the above criteria: $a=0$, $b=1$, $c=2$ and $d=3$. The maximum score for each tooth is 3. As the condition surrounding each tooth was recorded, data concerning one tooth can be studied, or a group of teeth, a quadrant or the whole mouth can be considered as a unit. Assessment of a gingival score of a quadrant from individual tooth scores can be made in one of several ways, such as the sum of the individual tooth scores or the sum of the tooth scores divided by the number of teeth present; but the most meaningful, when inflammatory conditions are considered, is the most severely affected area. This last has been used in this survey and the maximum score for a quadrant is therefore 3, which is the score of the most severely affected tooth. The score for the whole mouth is the sum of the quadrant scores with a maximum of 12 points.

FINDINGS

The Navajo children and Alabama children of European extraction have an almost identical gingival condition in terms of prevalence, severity, and incidence of gingivitis (Table 1). The children in the Navajo Mountain area have a slightly better, and the Montezuma children a slightly poorer, gingival condition than the Gardendale children; the new entries to the Indian School representing all areas of the Reservation have a somewhat better gingival condition than the white children in the Deaf and Blind school in Alabama. In the Navajo and Alabama groups also, the rise and fall in incidence of gingivitis with age is similar. The gingivitis scores rise from the age of 6 years to a peak period between the ages of 11 and 15 years, then fall to the age of 18 years. Exceptions to this pattern are the York children and the Navajo children who have been in boarding school for one or more years. In these little, if any, peak period is discernible during a continuous rise from an early age.

Although the prevalence, severity and incidence rate of gingivitis is similar there are many subtle differences between the condition of the gingiva of the Navajo and Alabama children. The most severely affected area of the mouth in the Navajo child

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is the upper molar region, whereas in Alabama children the upper or lower incisor regions are the most severely affected. In both groups the lower molar region is the healthiest (Table 2).

The incidence of calculus is much higher in the Navajo children (Table 3). It occurs subgingivally on the buccal surfaces of the teeth and is hard and dark in colour, a condition expected in much older individuals. In the new entries to school the incidence rises from 30 per cent in the younger children to 60 per cent in the older children, but the children from the remote areas have as low an incidence as the Alabama children. The type of calculus differs somewhat, for in the Alabama children the subgingival calculus on the buccal surfaces of the teeth in comparison is slight, soft and light in colour. The regions of the mouth in which calculus occurs is also different (Table 4). In the Navajo children by far the most common tooth to be affected is the upper first molar, 24 per cent of these teeth having calculus on the buccal surface, whereas only 1.4 per cent are affected in Alabama children.

As the buccal surface of the first upper molar is the most common site for calculus formation, an analysis was made of the surface conditions and the severity of gingival inflammation in the immediate vicinity of this particular tooth. Three surface conditions are recognized: the absence of deposits, the presence of soft deposits, and calculus. The percentage of children at each age in these categories and their average gingivitis score is shown in Table 5. The gingivitis score varies according to the condition on the tooth surface but changes remarkably little with age. The score is highest when calculus is present, nearly as high when there are soft deposits and lowest when the tooth is free of deposits. Differences in the overall average scores between various groups of children depend more on the percentage of children in the various categories than on a variation of the gingivitis score associated with a particular condition on the tooth surface. The presence or absence of gingivitis about the upper first molar, when the tooth is free of deposits or has calculus or soft deposits, is shown in Table 6.

Attrition of the teeth is common in Navajo children. The only attrition seen in the Gardendale children was in 28 per cent of lower incisors and 2 per cent of upper incisors. No facets were seen on the molar teeth of any of the Alabama children, but 51 per cent of all Navajo children had attrition of the first lower molar, and attrition was most marked in the children from the Navajo Mountain area. The most severely worn tooth was the first lower molar, and in the Navajo Mountain area 70 per cent of children had this tooth affected and 40 per cent of these were worn through to the dentine. Attrition of teeth in other parts of the mouth was 55 per cent of upper molars and 59 per cent of upper and lower incisors.

Many new entrants to school also had severe attrition but they did not come from any particular area of the Reservation. Only one-quarter of the children above the age of 16 years from all parts of the Reservation had no attrition of the lower molars, one-half showed facets and one-quarter had exposure of the dentine. The gingivitis score associated with the teeth with severe attrition was eight times less than that of the teeth with no attrition.

The caries experience of the Navajo children was low. A considerable amount of fluorosis was seen and there is fluorine present in much of the drinking water.

TABLE 2. COMPARISON OF GINGIVAL SCORES OF THE FOUR QUADRANTS (per 100 children, maximum score—300)

	Age	Upper molar	Upper incisor	Lower incisor	Lower molar
Navajo children	12	133	124	91	60
	13	125	114	91	59
Gardendale	10-14	59	62	78	48
Deaf and blind	10-14	115	128	110	52
York	10-14	120	148	147	118

The number of D.M.F. teeth of new entrants to school and of re-entrants who had lived on a modern-type boarding-school diet for one or more years are shown in Table 7. In the children who have been living at home the caries incidence increases very little during the 10 years between the ages of 11 and 20 years, but in boarding school children the incidence rises, as can be seen from Table 7, by approximately one D.M.F. tooth every 2 years.

DISCUSSION

It is generally accepted that an abundant supply of milk and fresh fruits and vegetables is necessary to maintain health. The diet of the Navajo is lacking in all of these, yet in spite of the marked differences in diet and in the mode of life, the Navajo children living in the most remote areas of the Reservation have a similarly good gingival condition to the Gardendale children who enjoy every modern advantage and considerably better gingival health than the children of York, Alabama. Even the amount of calculus on the teeth of these children is almost identical. The strictly limited diet of meat and bread is low in vitamin C, even when the ascorbic acid content of liver is taken into consideration; but this diet, which differs widely from the accepted normal standards and has apparently so many deficiencies, is not incompatible with good oral health. The Navajo children have some of the healthiest gingiva of any group so far examined. Presumably the Navajo obtains all the necessary requirements by eating viscera, head, brain, bone marrow, blood, feet and all fat, whereas dietary supplementation is necessary when only meat is eaten.

Calculus is the most severe form of local irritation and, although the Navajo children in general suffer a great deal of calculus, their gingivitis score is not high. The most common site for calculus formation in Navajo children is the buccal surface of the upper first molar and this is the area most severely affected by gingivitis. The next most severely affected area in the Navajo children, and the most severely affected area in the Alabama children, is the upper incisor region which is not a common site for calculus. In this particular area, therefore, calculus is not the cause of irritation. Soft deposits are associated with nearly as high a gingivitis score as calculus and in many children calculus is not present in the most severely inflamed area of the mouth. Soft deposits, therefore, assume considerable importance as they occur so frequently.

TABLE 3. PERCENTAGE OF INDIAN SCHOOL CHILDREN AND ALABAMA CHILDREN AT EACH AGE WITH SUBGINGIVAL CALCULUS ON THE BUCCAL SURFACE OF ONE OR MORE TEETH

Age	Indian school, new entries			Indian school, re-entries			Montezuma and Navajo mountain		Gardendale, Alabama		York, Alabama	
	No. of children	No. with calculus	% with calculus	No. of children	No. with calculus	% with calculus	No. of children	No. with calculus	No. of children	No. with calculus	No. of children	No. with calculus
11	19	6	31.6	16	6	37.5	8	1	31	2	33	1
12	264	93	35.2	75	25	33.8	15	0	52	7	46	3
13	133	55	41.4	111	43	39.1	11	2	8	3	34	3
14	84	36	42.8	102	41	40.6	14	1	20	1	33	4
15	49	24	49.0	90	34	37.8	4	0	5	2	47	9
16	34	20	58.8	59	26	44.1	9	3	3	1	42	8
17	31	10	32.3	23	8	34.8	6	1			42	6
18	23	13	56.5	8	4	50.0	5	2			11	1
19	10	3	30.0	4	1	25.0	6	1				
20	11	7	63.6	4	1		4	0				
Totals	658	267		488	188		82	11	119	16	288	35
Percentage with calculus	40.6			38.5			13.4		13.4		12.1	

TABLE 4. DISTRIBUTION OF CALCULUS IN NAVAJO AND ALABAMA CHILDREN IN WHOM CALCULUS IS PRESENT IN THE MOUTH, SHOWING THE PERCENTAGE OF SPECIFIC TEETH AFFECTED

Upper			Lower		
Tooth No.	Navajo children (%)	Alabama children (%)	Tooth No.	Navajo children (%)	Alabama children (%)
7	10	6	1	42	81
6	70	25	2	47	55
5	8	6	3	21	28
4	6	4	4	8	11
3	5	4	5	4	0
2	5	9	6	5	0
1	7	6	7	2	2

TABLE 5. CONDITIONS ON THE BUCCAL SURFACE OF THE UPPER RIGHT FIRST MOLAR AND THE GINGIVAL SCORE OF ITS MARGINAL GINGIVA (Maximum score—3)

	Age	No. of children	No deposits		Soft deposit		Calculus	
			%	Average score	%	Average score	%	Average score
New Entries	11	19	21	0.25	47	1.00	32	1.83
	12	262	15	0.55	59	1.13	26	1.37
	13	132	16	0.58	55	1.05	29	1.41
	14	84	19	0.44	52	0.90	29	1.37
	15	49	16	0.75	53	0.81	31	1.40
	16	34	15	0.40	35	1.16	50	1.48
	17	31	29	0.55	55	1.18	16	1.60
	18	23	13	0.33	48	0.75	39	1.33
	19	10	40	0.75	50	0.80	10	1.00
	20	11	18	0.00	36	1.00	46	1.80
Re-entries	12	16	31	0.60	38	1.16	31	1.20
	13	75	36	0.37	42	1.07	22	1.06
	14	111	40	0.77	38	1.14	22	1.41
	15	101	32	0.47	41	0.93	27	1.11
	16	90	51	0.59	25	1.18	23	1.33
	17	59	44	0.38	27	0.81	29	1.18
	18	23	48	0.36	30	0.85	22	1.60
	19	8	38	0.00	24	1.50	38	1.33
	20	4	75	0.30			25	1.00

The lower molar region is the most healthy area of the mouth, the most free from deposits and the site of the most severe abrasion of the teeth. Much of the Reservation is arid and sandy and attrition of the teeth is common in children from all districts.

The type of attrition found was a rounding of the tooth contours and crater formation in exposed dentine with many of the abraded surfaces out of occlusal contact. This type is caused by the friction of food. Few polished facets with sharp edges on opposing cusps were seen. A greater number of lower molars were worn by abrasion, yet the upper and lower molars erupt simultaneously and occlude with one another. A possible explanation is that the friction of food is greater against the lower teeth due to the manner in which the bolus of food is masticated and such friction does not allow deposits to accumulate.

TABLE 6. THE PRESENCE AND ABSENCE OF GINGIVITIS ABOUT THE UPPER RIGHT FIRST MOLAR IN 1140 NAVAJO CHILDREN, GROUPED ACCORDING TO CONDITIONS ON THE TOOTH SURFACE

	Gingivitis	No gingivitis
No deposits	126	181
Soft deposits	407	122
Calculus	287	17

TABLE 7. D.M.F. TEETH OF NEW ENTRANTS AND RE-ENTRANTS TO THE NAVAJO INDIAN SCHOOL

Age	New entries		Re-entries	
	No.	D.M.F.	No.	D.M.F.
11	19	0.52	—	—
12	264	1.02	16	1.37
13	133	1.12	75	1.62
14	84	0.71	111	2.74
15	49	1.14	102	3.68
16	34	1.23	90	3.55
17	31	1.22	59	4.47
18	23	1.73	23	3.82
19	10	2.20	8	3.25
20	11	0.91	4	4.50

Dental caries is low in all parts of the Reservation, particularly in those children of all ages just entering school. A low caries incidence might be expected from the high fluorine content of many of the well waters, and the high meat, and fat-laden carbohydrate diet contaminated by sand might also be a contributory factor. The diet, however, is altering rapidly with the higher standard of living and this would explain the apparent lack of increase of D.M.F. teeth with age in this static survey, for the younger children have already reached the caries incidence level of the older children.

The re-entrants to school have a higher D.M.F. rate than the new entrants, but a greater percentage have no deposits on the teeth. The oral hygiene instruction has

no doubt been instrumental in producing better gingival health but it has been unable to counteract the effects of the school diet on dental caries experience. The figures show an apparent average increase of one D.M.F. tooth every 2 years, but a longitudinal survey might prove the increase to be much greater, for the overall caries incidence rate of the Navajo children is rising.

Acknowledgements—This work was made possible by a National Institute of Health Grant No. D796 supplemented by a Grant from the School of Dentistry, University of Alabama. I wish to thank the staff of the U.S. Public Health Service, Indian Health Division, particularly at the Intermountain Indian School in Utah and the Rev. H. B. LIEBLER and his staff at the Saint Christopher's Mission in Bluff, Utah, for their assistance in the field survey, and the State of Alabama Department of Public Health.

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THE IMPLANTATION OF CELLS FROM THE ORAL MUCOUS MEMBRANE BY INJECTION NEEDLES

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Abstract—Experiments using human buccal mucous membrane excised at autopsy were carried out to determine what cells were conveyed into the deeper tissues by a fine injection needle. Material from the same source was also examined to determine the distribution in the mucous membrane of the normal bacterial flora. These organisms were found to be distributed superficially and not in the depths of the epithelium or its appendages. Examination of serial sections of needle tracks showed that bacteria from the surface were conveyed deeply into the tissues and that, in 211 out of 471 sections examined, a fragment of epithelium, consisting of several hundred cells, was also seen to have been implanted. Evidence from other work suggests that such implantations also occur clinically but that the oral tissues are able to deal with them without a severe reaction.

AMONG the hazards associated with injections, that of infection is probably the first that comes to mind. The sources of infection are, firstly, contamination of the solution injected, secondly, a dirty needle and thirdly, the organisms from the surface of the mucous membrane. Of these, the injection of an infected solution is the one most likely to have serious consequences, the severity of which will depend on the number and virulence of the organisms which the injected fluid contains. Providing that the solution injected and the needle are sterile, however, it may be asked whether there is any real risk of carrying organisms on the needle into the tissues. It may in fact be suggested that a fine (26 gauge) hypodermic needle will come into contact with few bacteria as it passes through the mucous membrane. Further, doubts may be felt as to the possibility of more than an occasional organism being conveyed deeply into the tissues on the smooth surface of a polished, stainless-steel needle.

The purpose of this investigation was, therefore, to determine whether such assumptions were justified. In addition, during the course of these experiments, observations were made on the distribution of the normal flora of the oral mucous membrane among the cells of the epithelium and in the epithelial appendages.

MATERIAL AND METHODS

The experiments were designed with the object of examining complete needle tracks passing through the mucous membrane and into the underlying connective tissue, in order to determine what material might be conveyed along this path and how deeply it might be deposited. Though it was desirable to use living material, the problem of excising from the mouth the volume of tissue necessary to ensure

that the whole of a needle track was included made this method impractical. Furthermore, to find and section a single needle track in such a bulk of tissue after the distortion of the tissue by fixation would be very difficult. An alternative was to use human autopsy material and to make the needle tracks into tissue already excised. A possible objection to such a method is that there might be, on the oral mucous membrane of the cadaver, excessive numbers of bacteria which would be likely to give misleading results when compared with clinical conditions. Smears of the superficial oral squames were therefore taken from living subjects and from autopsy material. These were fixed in Schaudinn's solution and stained by Gram's method, and a comparison was made between the number of organisms present on the epithelial cells of living and dead subjects.

A second objection to the use of autopsy material is that after death the tissues gradually become friable and separation of the layers of the epithelium may occur. The possible consequences of this are discussed later. However, sections in which it was apparent that the surface cells had become stripped off were discarded.

Ten pieces of buccal mucous membrane, together with as great a thickness as possible of the underlying connective tissue, were excised from autopsy specimens. The pieces of mucous membrane were then cut into strips 3-4 mm wide to form fragments of convenient size for sectioning. Needle punctures into these strips were then made, using sterile, stainless-steel needles of 26 gauge. The needles were driven in through about three-quarters of the thickness of the specimen and then withdrawn. In order to increase the number of organisms present, so that they might be more easily seen, the specimens were next incubated at 37°C in moist conditions, for 3-7 hr (LOVELL, 1945).

In two of the initial experiments of this sort, the needles were first dipped into a dilute aqueous suspension of carmine. This served two purposes in that it showed whether material of a similar particle size to bacteria could be carried in on the surface of the needle, and in addition, whether the carmine made the needle track visible in the block which thereby could be correctly orientated for section cutting. In spite of this advantage, however, the use of carmine had to be given up as some of the particles could easily be confused with Gram-negative bacteria.

After incubation, the specimens of mucous membrane were fixed in formol-saline and paraffin blocks prepared. Serial sections were cut at right angles to the surface, with the long axis of the specimen in the plane of the section, and stained either in haematoxylin and eosin, or by Jensen's modification of Gram's method.

The sections were examined for the presence of bacteria on the mucous membrane and in the needle tracks, for the distribution of carmine in the early experiments mentioned, and for the presence of epithelial cells from the surface, in the needle tracks.

RESULTS

A. *Normal distribution of bacteria on the mucous membrane*

Smears made from scrapings of the oral mucosa of living subjects show many bacteria adherent to the superficial squamous cells. These bacteria on individual

cells may amount to a few dozen, or to many hundreds completely obscuring the nucleus and contents of the cell. Separate groups of organisms lying unattached to squamous cells may also be seen in such a smear.

Smears taken from the oral mucous membrane of cadavers show no greater numbers of bacteria than those taken from living subjects. This may be accounted for partly by the fact that the cadavers were edentulous, and partly by the handling that the mucous membrane receives after death, whereby much of the superficial debris is rubbed off. In addition, because of the gradual tendency of the tissues to disintegrate after death, the more superficial cells of the epithelium and their adherent bacteria may separate and be lost.

Sections of mucous membrane inevitably show fewer organisms on the superficial cells than appear in smears, as these cells are seen "edge on", and only those organisms appear that lie in the strip 5-7 μ thick which forms the section.

In all specimens, the bacteria were found to be superficially situated on the epithelial cells. No organisms were seen situated deeply in the epithelium, in the mucous glands or in their ducts. Bacteria were sometimes seen on the squamous cells where the epithelium is invaginated to form the orifices of the ducts of the sebaceous glands which, as MILES (1958) has shown, are plentiful in the oral mucous membrane.

B. *Material seen in the needle tracks*

When needles had been inserted through the surface, the following results were obtained.

(1) Fragments of epithelium were found at varying depths in the underlying connective tissue along the needle tracks.

This was observed in 211 sections out of a total of 471 that were examined. In many of these sections, due to distortion of the tissue during fixation, no parts of the needle tracks, or only very short lengths, were present.

The fragments of epithelium were of varying size and were seen at any point along the needle track from its point of entry into the corium, down to what appeared to be its termination.

The epithelium at the point of entry of the needle showed a gap where cells had been lost, and varying degrees of damage to the adjacent cells which had not been displaced. Where the needle had entered obliquely, a considerable length of the more superficial part of the epithelium was sometimes seen to have been raised from its deeper attachment.

(2) Specimens in which the needle had been first dipped into a suspension of carmine showed that the particles were carried along the whole length of the needle track.

(3) Specimens which were incubated after sterile needles had been inserted, showed dense clumps of bacteria at varying depths in the needle tracks. These bacteria were seen both along the margins of the tracks and around implanted epithelial cells.

No bacteria were found to have been displaced from the surface of the specimen anywhere into the deeper tissue during the experiments, except along the needle tracks.

DISCUSSION

The finding that the bacteria of the oral mucous membrane were located almost solely on the superficial squames is unexpected in that it is in contrast with the distribution of the bacteria of the skin. LOVELL (1945) showed that the bacteria of the skin were present in considerable numbers in the ducts of the sebaceous glands and that in this situation the bacteria were unaffected by the application of antiseptics or by cleansing the surface. It is possible that for the oral bacteria, unlike certain of the skin bacteria, the secretions of the sebaceous glands are inimical to their growth.

From the practical point of view, the superficial distribution of the oral bacteria suggests that they are accessible to antiseptics; on the other hand, contamination of the disinfected surface by saliva is likely to occur.

It is clear from these experiments, however, that if no attempt is made to disinfect the surface of the mucous membrane, a needle will be contaminated as it passes through the surface and will convey bacteria along the whole length of its track. Further, a mass of epithelial cells together with their accompanying bacteria may also be implanted. Under the conditions of these experiments, the organisms proliferated freely, but in life it is probable that this multiplication would be limited by the defence mechanisms of the body. Nevertheless, in these experiments the number of organisms seen in the deeper tissues after so short a period of incubation is striking.

Since autopsy material was used in these experiments it is possible that some autolysis had occurred so that the tissue might have become sufficiently friable to allow fragments to be torn away and implanted by the injection needle more easily than in living tissue. There seems, however, to be good reason to suppose that the implantation of epithelial cells which occurred in these experiments also occurs clinically. MÜLLER (1950) who carried out somewhat similar experiments on the oral mucous membrane appears to reach this conclusion, whilst in the case of the skin GIBSON and NORRIS (1958) have shown that injection needles regularly core-out plugs of epithelium.

It is difficult to assess the quantity of epithelium that may be implanted but in four specimens the numbers of nuclei in the fragments were counted and were found to range from 92 to 140. Assuming, for the sake of calculation, that the fragments were spherical and that the section passed through the diameter, then the number of cells in such fragments would be approximately 600-1200.

Even though the superficial squamous cells form only a proportion of these implanted masses, some idea can be gained of the number of organisms that can be carried in by a needle in this way, when it is recalled how densely an individual squame may be populated by bacteria.

The implantation of fragments of epithelium, even if uninfected, emphasizes the well-recognized dangers of making injections through inflamed tissues, because these cells would form in the tissues an avascular mass which could serve to protect proliferating bacteria from phagocytosis.

The fate of the epithelial fragments is not clear. Since implantation dermoids are not a known consequence of injections in the mouth it is assumed that the epithelial

cells must die, perhaps as a result of the toxicity of the local anesthetic solution. GIBSON and NORRIS (1958) suggest that the injection fluid, and possibly blood clot, are inimical to the survival of these minute epithelial grafts.

In spite of the evidence that many bacteria and epithelial cells may be implanted by an injection needle, it is a matter of common observation that a clinically apparent infection is a rare consequence of the many injections that are carried out in the mouth. The oral tissues are able, therefore, to deal with the cells that are implanted in this way. This is not, however, to say that there is no reaction to such foreign matter merely because there is no obvious acute inflammatory condition results. It is a matter of interest, therefore, to know how much of the after-pain, which seems to be an accepted consequence of local anaesthetic injections, is due to an inflammatory reaction in response to the introduction of bacteria and of epithelial cells into the tissues.

Acknowledgements—I am most grateful to Professor H. A. MAGNUS, Director of the Department of Pathology, and Dr. A. C. CUNLIFFE, Head of the Department of Bacteriology, for their generous help and for their criticisms of this paper. My thanks are also due to Miss GLADYS STEBBENS for preparing the sections.

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THE IMPLANTATION OF CELLS FROM THE ORAL MUCOUS MEMBRANE BY INJECTION NEEDLES

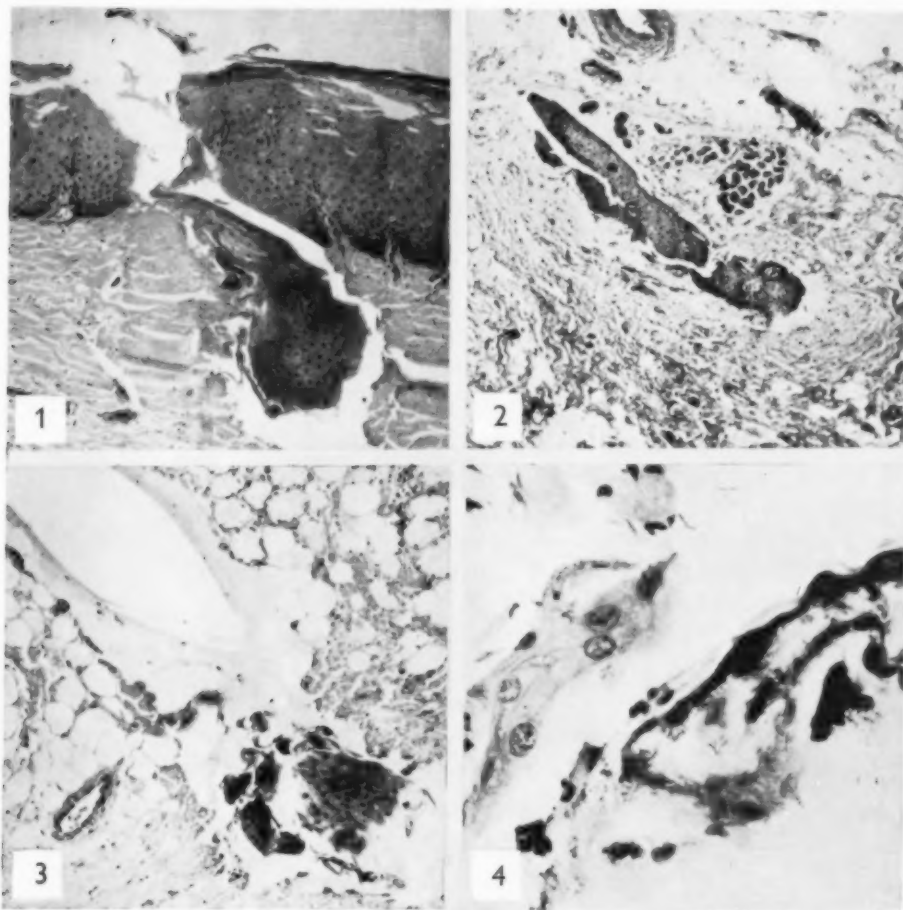


FIG. 1. Section showing the point of entry of a needle through the mucous membrane and a large fragment of epithelium implanted just beneath the surface. Haematoxylin and eosin. $\times 18$.

FIG. 2. Section showing a large, elongated fragment of epithelium deeply implanted in the submucous connective tissue. Haematoxylin and eosin. $\times 100$.

FIG. 3. Section showing a relatively small fragment of epithelium implanted at what appears to be the termination of a needle track. Haematoxylin and eosin. $\times 180$.

FIG. 4. Epithelial cells implanted in the tissues together with dense masses of proliferating bacteria. Haematoxylin and eosin. $\times 210$.

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SOME OBSERVATIONS OF TOOTH ENAMEL SURFACES

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Abstract—The relatively flat portions of the labial surfaces of human central incisor teeth were polished with various abrasives, in an attempt to produce consistently reproducible surfaces for the comparison of the effects of various treatments. The natural tooth surfaces were so masked by irregularity that the effects of these treatments were difficult to interpret. It was found that when these surfaces were polished by high lustre-producing abrasives, such as tin oxide or levigated alumina, a reproducible surface was formed. These abrasives obliterated all traces of the rod ends, producing the appearance of a Bielby layer. This surface resisted the action of weak acids and did not respond normally to the application of fluorides. When chalk was used as the abrasive another characteristic and reproducible surface was formed. The rod ends and inter-rod areas of the tooth were clearly visible and the surface responded evenly and reproducibly with weak acids and fluorides. Portions of such surfaces were treated with fluorides and oxalates and the entire surface then etched with 0.1 M lactate buffer of pH 4.5, and the boundary zone between the treated and untreated areas was examined. The protected areas of the surface were clearly visible.

Attempts were made to produce replicas of carious lesions in the enamel at various increments of depth. It was found that replicas could be obtained near the depth of maximum penetration. These replicas showed each rod protruding, with the inter-rod substance decalcified.

INTRODUCTION

THE enamel of teeth is the hardest substance in the body. It has a very definite structure, a composition which is constant within narrow limits, and it resists most substances that are normally found in the mouth, with the exception of acids. When clean and dry it may be rather dull but it can be polished to varying degrees of brilliance depending upon the polishing agent used. Chalk produces a sort of velvet finish without lustre. Tin oxide, levigated alumina and insoluble sodium metaphosphate produce a brilliant lustre. The various calcium phosphates produce a lustre somewhere between that produced by the chalk and the brilliant lustre of the levigated alumina. When the polished tooth surface is wet with saliva, the differences in degree of polish obtained from the various polishing agents generally diminish and the clean wet surface appears highly polished.

The enamel of the natural tooth surface under high magnification may appear smooth, gnarled or corroded, but when polished with any of the abrasives mentioned above will generally yield a smooth surface. This surface must first be penetrated for the formation of a carious lesion. Under ordinary circumstances, when an extracted tooth is etched with pure dilute acids the initial etch indicates a more rapid solution of the rods. Further exposure to acids destroys all traces of structure. In the development of a smooth-surface carious lesion, it would appear that the cementing substance dissolves first.

When powdered enamel is treated with fluorides, it resists the attack of acids. This action should, presumably, be detectable on intact teeth.

In view of the importance of the enamel surface, it was thought that an electron microscopic study of it and its response to various treatments would be of interest. There have been a number of publications depicting enamel surfaces and acid-etched surfaces, but none has shown the comparative effect of various polishing agents, nor have any demonstrated the early stages of enamel caries by the methods herein shown (SCOTT and WYCKOFF, 1946; SCOTT, USSING, SOGNAES and WYCKOFF, 1952; KENNEDY, TEUSCHER and FOSDICK, 1953; LENZ, 1956; SCOTT and ALBRIGHT, 1954; MATSUMIYA, TAKUMA, TSHUCHIKURA and OKADA, 1956). This paper deals with the effect of polishing agents, acids and fluorides on the enamel surface. In addition, the appearance of early caries of the enamel is presented.

MATERIALS AND METHODS

The preparation of replicas

In these experiments only the relatively flat labial surfaces of central incisors were used.

In each case a replica of the surface was taken by means of polyvinyl alcohol. The alcohol was applied to the surface and allowed to set for approximately 4 hr. When it had attained a suitable consistency, it was stripped from the surface. The replica surface was then covered with a thin film of Formvar and allowed to dry. It was then floated on water with the polyvinyl alcohol portion in contact with the water. The polyvinyl alcohol dissolved, leaving the thin Formvar replica floating on the surface. The replica was placed on a steel grid, allowed to dry and shadowed with chromium, after which it was examined in the electron microscope. The advantage of this indirect replication is that the thick, pliable, polyvinyl alcohol film may be stripped from undercuts without tearing.

The preparation of the surface

The natural tooth surface. The tooth was cleaned with a toothbrush and water, set in quick-setting acrylic resin for ease of handling, and the surface moisture removed.

The polished tooth surface. The tooth was set in quick-setting acrylic cement for ease in handling. It was then polished or buffed on a metallurgical polishing wheel, using the appropriate abrasive slurry on a velvet pad.

Acid etch. In order to provide a standard surface for comparison, the tooth set in acrylic resin was buffed with heavy chalk. This yielded a surface that could be duplicated at will. It was then immersed in acid or buffer solution for the appropriate time, washed with water and dried. If the tooth was to be treated with a protective agent, one half of the standard surface was covered with polyvinyl pressure-sensitive tape and the uncovered half treated with the agent for the appropriate time. The surface was then washed and the tape stripped off and washed again. Then the whole surface was etched with acid in the usual manner.

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Early carious lesions

The method used was originally devised by POLARA (1953). The tooth was set in acrylic resin, as previously described, with the early carious lesion or "white spot" exposed. An attempt was made to obtain a satisfactory replica. In most cases the "holes" corresponding to the cementing substance were so deep that intact Formvar replicas could not be obtained. If the Formvar replicas were not intact, the surface of the lesion was lightly stroked with a fine "gold file" to remove the outer layer. Another replication was attempted, and if not successful the area was again stroked with the file to remove another layer. This procedure was repeated until intact replicas were obtained. By the use of this method several replicas of the deeper regions of early enamel caries may be obtained. In no cases could replicas be made if the empty spaces between the rods were large enough for microbial invasion.

RESULTS AND DISCUSSION

The results are shown in the following micrographs. Figs. 1-3 show various normal tooth surfaces. Actually almost all possible variations from smooth surfaces to microscopic eroded areas may be observed. In most cases these variations may be observed in different areas of the same tooth. In all cases the surface of the teeth appeared normal to the naked eye.

Because of the many variations of normal enamel surfaces it is difficult to interpret the results of various treatments; hence, it is imperative that a standard surface be prepared. Fig. 4 shows such a standard surface. This type of surface may invariably be prepared on a sound tooth by buffing with "heavy" chalk. Each rod end is visible and it appears that the cementing substance is eroded faster than the rod substance. This surface reacts characteristically to acid etch and to the absorption of tin fluoride or neutral sodium fluoride. When dry, this surface has a dull velvety sheen.

When the tooth is buffed with levigated alumina, tin oxide, insoluble sodium metaphosphate or calcium sulphate, the dry surface takes on a brilliant polish. This type of surface can be prepared on a natural tooth surface or on a chalk-buffed surface. The latter usually requires less buffing. Fig. 5 shows such a surface. All abrasives that produce a high lustre yield such a surface and micrographs of each cannot be differentiated. This type of surface can also be produced at will and hence might be considered a standard surface. However, this surface is not readily etched at pH 4.5. The amorphous surface, with all rod ends obliterated, appears to offer some degree of protection against acids and hence cannot be used as a "standard surface". When the highly polished, amorphous surface is buffed with chalk, the typical surface (Fig. 4) is produced.

When the chalk-buffed surface is etched with 0.1 N hydrochloric acid for 5 sec (Fig. 6) a characteristically mild decalcification is produced. This type of surface may be produced with weaker acids or with buffered acid solutions. A 0.1 N lactate buffer of pH 4.5 will ordinarily yield such a surface with a 15 min etch. However, with such mild etching agents there is considerable variation from tooth to tooth. In order to obtain a satisfactory etch, the time of immersion in the acid solution will vary from 10 to 20 min (Fig. 7). A highly polished surface such as is shown

in Fig. 5 may resist etching with 0.1 M lactate buffer of pH 4.5 for 30 min. Much of the variation in the pattern of the etched tooth surfaces can be eliminated if the surface is previously prepared by buffing with chalk.

When a portion of the standard surface is treated with neutral 2% sodium fluoride or a neutral sodium fluoride dentifrice for 2½ hr after which the entire surface is etched for 15 min with a lactate buffer, the treated portion is protected against etch (Fig. 8). However, if a surface is treated with acid 2% sodium fluoride of pH 3, calcium fluoride globules always form (Fig. 9). This layer is readily brushed off. When 4% tin fluoride of pH 3 is used, or if a stannous fluoride dentifrice is used as the treatment, the surface is amply protected against 0.1 M lactate buffer of pH 4.5 (Fig. 10). Sodium oxalate also appears to offer a mild protective action (Fig. 11).

When a carious lesion is visible to the eye, it has gone too far for replication. However, if the surface layers are removed by a fine "gold file", the rods are scraped off to the point that the organic and cementing substances will support the replica. Figs. 12 and 13 show such cavities. It will be noted that the cementing substance is completely decalcified, leaving the rods without rigid support. This supports the observations made with the light microscope. It should be noted that such micrographs can be obtained from any enamel caries, providing the process has not penetrated the dentine. The primary difference between such lesions is the depth at which the organic matter in the cementing substance will support the replica.

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FIG. 1. Clean untreated enamel surface. $\times 9300$.

FIG. 2. Clean untreated surface. $\times 13,600$.

FIG. 3. Clean untreated surface. $\times 9300$.

FIG. 4. Clean surface, buffed with Sturges heavy chalk. Note each rod end is clearly visible. $\times 9300$.

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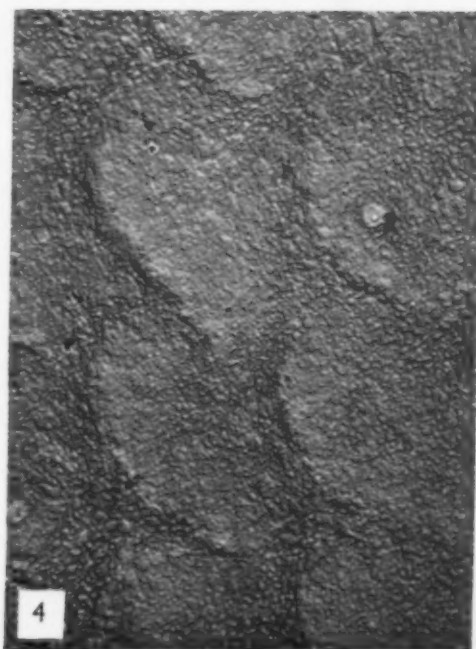
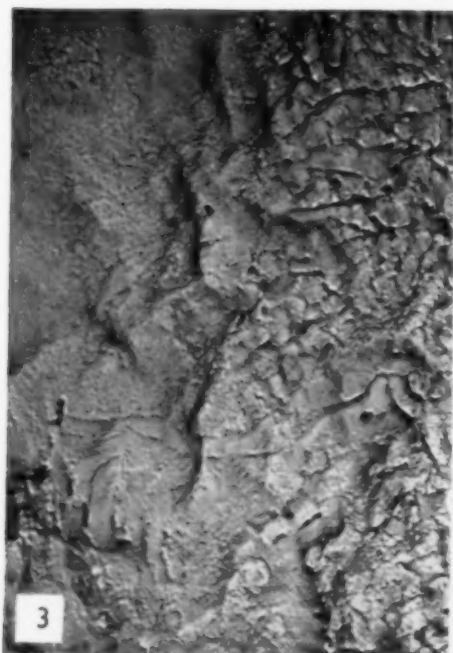
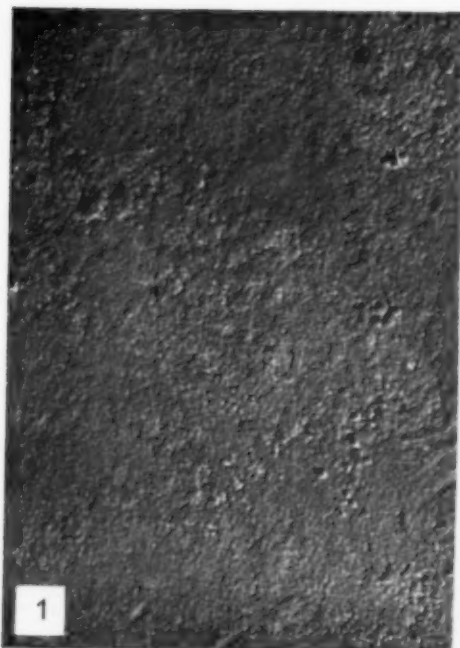


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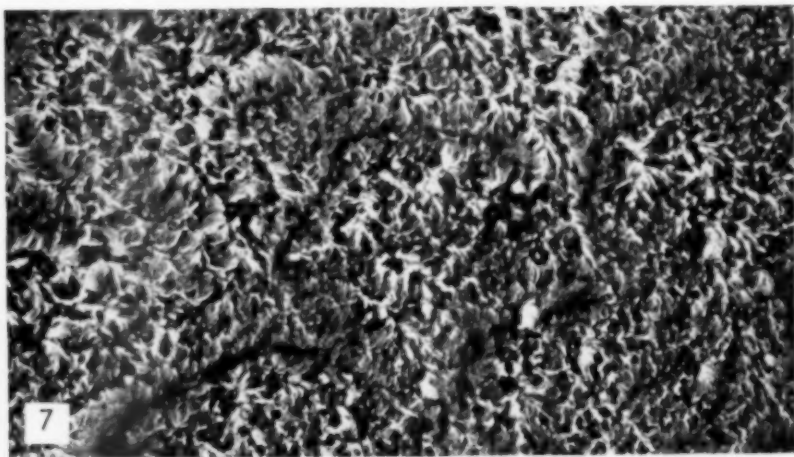
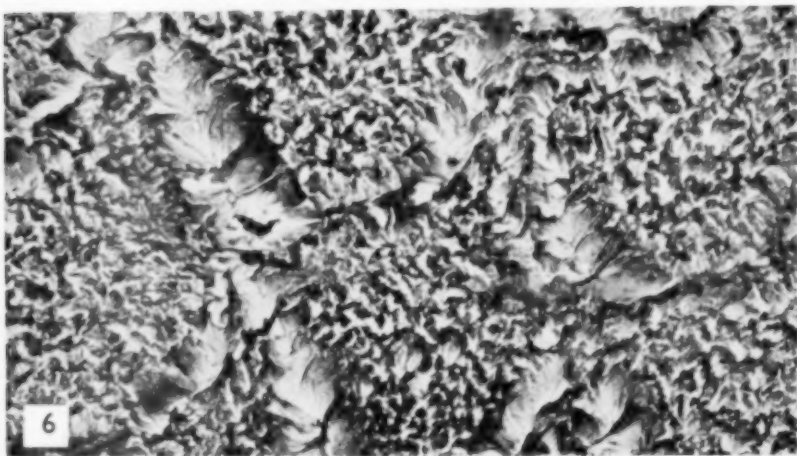


FIG. 5. Surface buffed with levigated alumina. $\times 24,000$. Surfaces polished with tin oxide, insoluble metaphosphate or calcium sulphate yield the same type of structure.

FIG. 6. Chalk-buffed surface etched 5 sec with 0.1 N HCl. $\times 9300$.

FIG. 7. A chalk-polished tooth etched 15 min with 0.1 N lactate buffer of pH 4.5. $\times 9300$.

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FIG. 8. The boundary between a chalk-polished surface (left) and a chalk-polished surface treated with a 2% sodium fluoride dentrifice for $2\frac{1}{2}$ hr (right). The whole surface was then etched for 15 min with 0.1 N lactate buffer of pH 4.5. $\times 13,600$.

FIG. 9. Chalk-polished surface treated with 2% sodium fluoride solution of pH 3 for 2 hr. $\times 20,000$. Note globules of calcium fluoride.

FIG. 10. The boundary between a chalk-polished surface (below) and a chalk-polished surface treated with stannous fluoride dentrifice for $2\frac{1}{2}$ hr (above). The entire surface was then etched with 0.1 N lactate buffer of pH 4.5 for 15 min. $\times 9300$.

SOME OBSERVATIONS OF TOOTH ENAMEL SURFACES

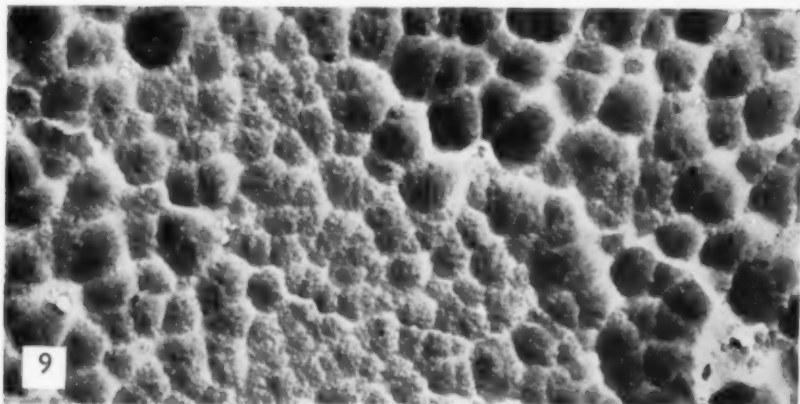
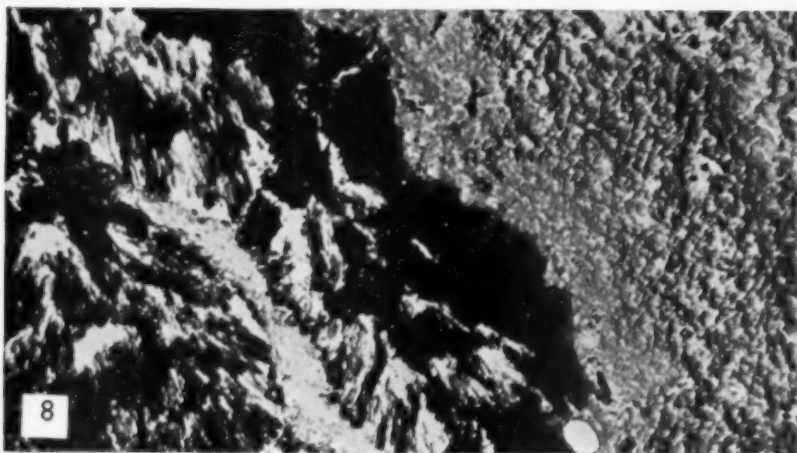


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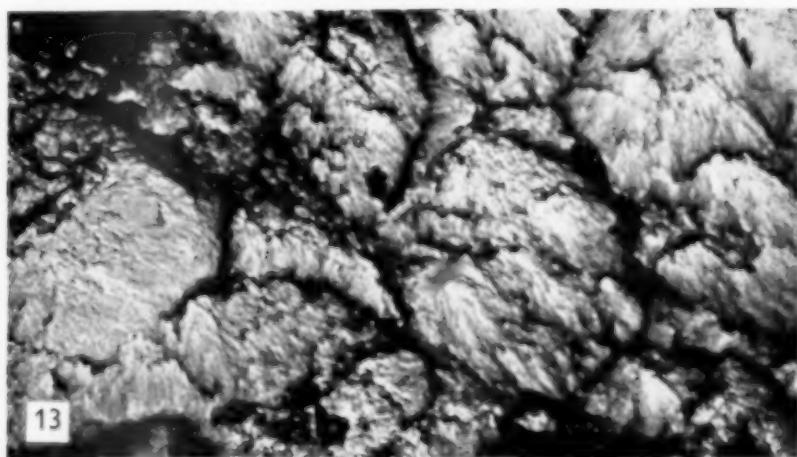
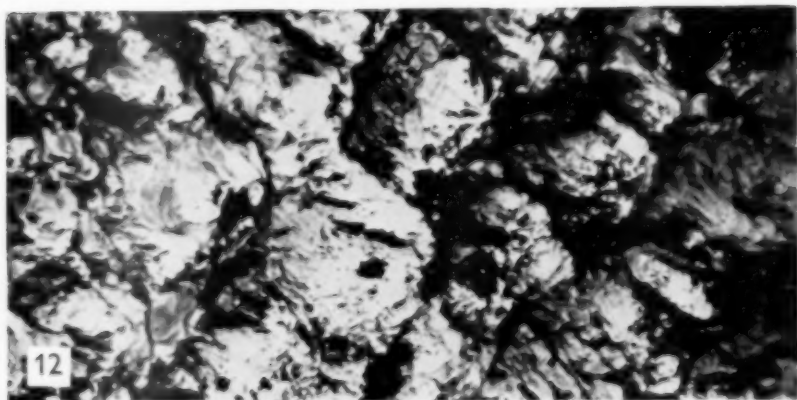
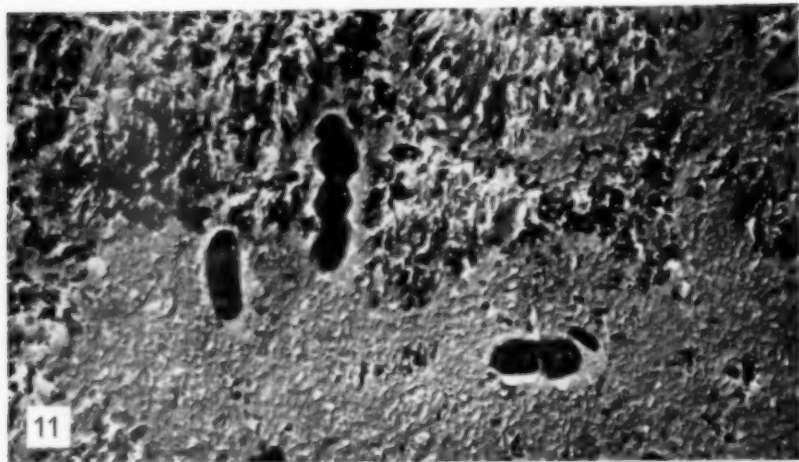


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FIG. 11. The boundary between a chalk-polished surface (above) and a chalk-polished surface treated with 2% sodium oxalate solution for $2\frac{1}{2}$ hr (below). The entire surface was then etched for 15 min with 0.1 N lactate buffer of pH 4.5. $\times 9300$.

FIG. 12. Natural carious lesion of the enamel, near the bottom of the lesion. Note rods with inter-rod substance dissolved. $\times 9300$.

FIG. 13. Natural carious lesion of the enamel, near the bottom of the lesion. $\times 13,600$.

SOME NOTES ON THE SOLUBILITY OF ENAMEL AND DENTINE IN ACID

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Abstract—The reaction between 0.2 M acetic acid buffer, pH 4.70, and finely powdered human enamel and dentine rapidly reaches equilibrium although the tooth mineral may not be fully decalcified and though the change in pH of the buffer solution may be small. The solubility in the buffer solution varies with the amount of solid material present and is inversely related to the fluoride content of the sample, the dependence of the solubility on solid:solution ratio decreasing as the fluoride content is increased. The solubility at a fixed solid:solution ratio is directly related to the molality and indirectly related to the pH of the decalcifying buffer. Because the solubility is dependent on these several uninterrelated variables, the pH of the acid-mineral solution does not necessarily indicate the extent of the decalcification that has taken or is about to take place.

Calcium fluoride at equilibrium in suspension with fluoridated tooth mineral has no appreciable effect on its solubility in acid and under these conditions the free fluoride concentration is low.

INTRODUCTION

The object of the present paper is to present the results of some studies on the acid decalcification of normal and artificially fluoridated enamel and dentine which show the extent of, and the need for, the control of some of the less obvious variables involved in this reaction.

Aberrant properties of the mineral

Many reports appear in the literature from several laboratories of the results obtained on decalcification of enamel, dentine and hydroxyapatite by a variety of acids over a range of pH values (HOLT, LA MER and CHOWN, 1925; BRUDEVOLD, 1947; MUHLER and VAN HUYSEN, 1947; RAE and CLEGG, 1948; MANLY and BIBBY, 1949; HODGE, 1951; SUESS and FOSDICK, 1951; JENKINS, ARMSTRONG and SPIERS, 1952; LITTLE, BUONOCORE and SOMMER, 1954). Many of these results are at variance with each other yet it appeared that similar, or almost similar, conditions had been present in each case. It is thought that the presence of trace quantities of unidentified material is one of the factors leading to the apparent variability in the solubility of tooth substance. In certain instances where the presence of foreign ions had been shown, a difference in solubility has been found between this and normal enamel under otherwise identical conditions. It has only recently been recognized that the properties of hydroxyapatite, the mineral of calcified tissue, do not follow the simple laws of chemistry relating to solubility. Ionic compounds usually show a constant solubility product under any particular set of conditions independently of the amount of excess

solid present: if excess solid is not present the whole of the solid dissolves. This is not so for hydroxyapatite, where the solubility is a function of several variables which include, in the presence of excess solid, the following:

- (i) the relative amounts of solid and decalcifying solution present in the system
- (ii) the composition of the solution in terms of ions both normal and foreign to the hydroxyapatite lattice
- (iii) the pH of the system.

This apparent deviation is thought to be the result of the small size of the crystals of hydroxyapatite. Both synthetic and naturally occurring hydroxyapatite crystals are of colloidal dimensions (*circa* $200 \times 30-70$ Å), and therefore possess a high surface area to mass ratio. The consequent high surface energy or escaping tendency of the ions of such small crystals is the cause of their aberrant properties.

In deriving the relationship for the solubility of an ionic solid, the activity of the solid is presumed to be constant. This is quite justified for large, pure crystals. The activity of a pure solid in contact with a solvent is a measure of the tendency of the ions of the solid to escape from the crystal surfaces. At a fixed temperature this escaping tendency is a fixed quantity and is equal to the number of ions returning by collision to the crystal and the solid has a fixed solubility. Opposing this thermal escape are the interionic attractions of the ions in the crystal lattice. For large crystals this also is a constant. In very small crystals the surface ions are less restrained by the attraction of the underlying ions, and hence have a higher escaping tendency than those from an "infinitely large" crystal (NEUMAN and NEUMAN, 1958). This latter attraction of the underlying ions varies with the size and composition of small crystals and results in them having a variable activity. If the activity of the solid is not constant, then the activity of the ions in solution is no longer a constant and the normal ionic product or solubility product of the ions in solution from the crystal is also no longer a constant.

Choice of material

It was decided to use enamel and dentine in a finely divided form. The advantage of using a powdered material rather than whole teeth is twofold. First, a large homogeneous sample may be prepared and its properties studied under different conditions without having to take into account the individual variability of each tooth, i.e. the variables are then imposed and controlled. Second, because of the high fraction of the powdered tooth immediately available for chemical reaction the amount of material taking part in the reaction is increased and equilibrium conditions are established relatively quickly.

Decalcification in the presence of, and by, foreign ions

In many experiments designed to show the solubility of enamel in acid after prior or simultaneous reaction with various salts, it has not always been fully recognized that the same salts may undergo chemical reaction with the tooth material to form a separate solid compound or, during the course of the pH changes taking place on reaction of the acid with the enamel, a new compound may be deposited out of

the solution. This is of particular significance in experiments in which the extent of decalcification has been estimated before equilibrium has been established, i.e. in a rate study or where the extent of decalcification has been estimated by the change in weight of the sample before and after the acid treatment. As has already been pointed out by RAE and CLEGG (1948) "any salt whose anions form an insoluble calcium salt (e.g. ammonium oxalate) or whose cations form insoluble phosphate salts (e.g. lead nitrate, barium nitrate) will protect the teeth and reduce the apparent solubility of tooth enamel and calcium phosphate". Other ions (e.g. Be^{2+} , Sn^{2+} , Pb^{2+}) form polymeric colloidal compounds at certain pH values and form a temporary mechanical barrier to acid attack on the crystal surfaces. This barrier, which is also formed in the previous instance, will alter the amount of tooth material dissolved in any system that has not had time to reach equilibrium, though its effect on reducing the actual solubility may be nil. Also, any experiments in which the extent of decalcification is measured by the weight loss after treatment of the sample in acid do not take into account the weight of this new compound and some authors using this technique have reported an actual weight increase under these conditions (MUHLER and VAN HUYSEN, 1947; MANLY and BIBBY, 1949). In most instances this extra compound has no ability to reduce the solubility of the tooth material and it has been found that some compounds which reduce the apparent acid solubility *in vitro* produce no significant reduction in caries when tested *in vivo* (LAZANSKY, 1947). The formation of this extra salt due to the double decomposition reaction between the tooth mineral and one or both the ions of the salt actually leads to some decalcification of the tooth material. This becomes obvious when it is realized that the tooth releases both calcium and phosphate ions whether the new insoluble compound is formed with either ion, as the crystal lattice must maintain electrical neutrality. The mass action law shows that the higher the concentration of the added salt under these conditions the greater the extent of the decalcification. In one experiment (LEACH, 1959), 15 per cent decalcification was achieved by shaking powdered dentine in a concentrated solution of sodium fluoride at an alkaline pH.

There are instances, however, in which one or more of the ions of the salt are incorporated into the crystal lattice or are chemically absorbed at the crystal surfaces without forming a new compound as a separate phase. Fluoride ions, incorporated into the lattice to form fluorapatite, will reduce the solubility of tooth mineral; carbonate, nitrate, magnesium and sodium increase the effective solubility; plumbous, strontium and uranyl ions are known to substitute for the calcium ion but their effect on solubility is not established (NEUMAN and NEUMAN, 1958).

Fluoride

It is well established that incorporation of fluoride into the crystal lattice of hydroxyapatite reduces its solubility in acid. The extent of this reduction, however, is not well established, particularly as several experiments have been carried out at high concentrations of fluoride under conditions which lead to the formation of calcium fluoride and decalcification of the mineral prior to the decalcifying action of acid and before equilibrium has been reached.

In general, the mass action law shows that for two different solid ionic compounds in suspension in a liquid containing a common ion, the concentration of the common ion dissolved in the solution will be very nearly equal to that obtained on shaking the more soluble solid alone in the same solution. At a low pH, tooth mineral is more soluble than calcium fluoride and the concentration of calcium, the common ion, will be virtually independent of the presence of calcium fluoride. At higher pH values the solubility of tooth mineral decreases and at neutral pH calcium fluoride is more soluble than the tooth mineral and the concentration of calcium in solution is that expected from calcium fluoride if present alone in the solution.

However, in the case of fluorapatite in which the fluoride is incorporated into the crystal lattice of the tooth mineral the solubility is then a function of the fluoride concentration of the particular fluorapatite and is less than hydroxyapatite containing a smaller concentration of fluoride.

The high concentration of calcium, released on treatment of the tooth material with acid, depresses the concentration of fluoride ion in solution. This is because the ionic product $[Ca^{2+}] \times [F^-]^2$ cannot exceed a certain value. Of the various possible entities of fluoride in solution (F^- , HF , HF_2^- and CaF^+), a definite fraction of the total fluoride is present as free fluoride ion, F^- . Even at a low pH, where HF and HF_2^- are formed, there is still a sufficient fraction of the total fluoride present in solution as free fluoride to keep the total fluoride in solution at a low value.

The problem to be investigated

The purpose of the investigation was to examine the effect of certain variables upon the acidic decalcification of normal and fluoridated human enamel and dentine. The investigation was planned in four parts, as follows.

Firstly, an investigation of the acid decalcification reaction under equilibrium conditions.

Secondly, an investigation into the relationship between the quantity of tooth material dissolved by acid and the quantity of tooth material initially present in suspension.

Thirdly, an investigation of the effect on the decalcification reaction of different strengths of acid, either at the same or at different pH values.

Fourthly, the effect on the decalcification reaction of the presence of fluoride, either incorporated into the hydroxyapatite crystal lattice or when present as calcium fluoride.

MATERIALS AND METHODS

Preparation of enamel and dentine samples

The method used has been fully described elsewhere (LEACH, 1956, 1959). Enamel and dentine from freshly extracted, sound teeth was ground to a fine powder in a ball-mill at low temperature and separated by the method of MANLY and HODGE (1939). By this procedure some 50 g of enamel and 150 g of dentine were obtained, the majority of the particles having a diameter of 20 and 30 μ for the enamel and dentine respectively.

Treatment with fluoride

The powdered samples were shaken at a concentration of 1 per cent in different concentrations of sodium fluoride solution for 6 hr at room temperature ($20 \pm 2^\circ\text{C}$). The samples were then separated from the solution by centrifugation and washed five times in separate consecutive amounts of distilled water at a concentration of 1 per cent, the samples being separated from the supernatant solution by centrifugation at each washing. This procedure was to remove any excess fluoride, that may have been present, from the original sodium fluoride solution. Control samples were washed a similar number of times in distilled water and all samples were dried to constant weight over silica gel.

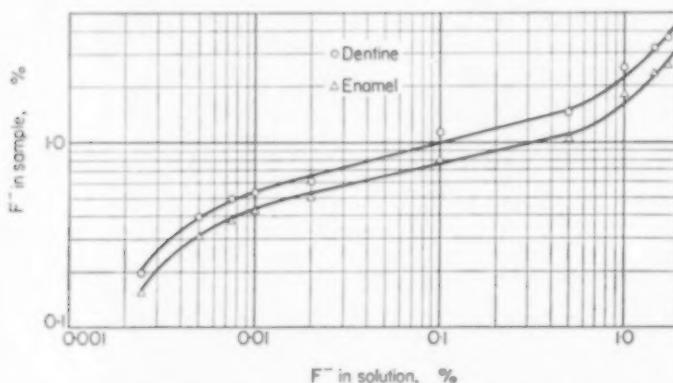


FIG. 1. The uptake of fluoride (percentage weight of sample) by 1 g samples of enamel and dentine shaken for 6 hr at room temperature in 100 ml of fluoride solution (percentage concentration) as sodium fluoride. Both scales are logarithmic.

The concentration of fluoride in any particular sample (expressed as percentage weight) after shaking in the various fluoride solutions is shown in Fig. 1. The various fluoridated samples will subsequently be referred to, and identified by their fluoride content. Material that had not come into contact with fluoride solution will be referred to, where necessary, as normal enamel and normal dentine.

Decalcification

Acid Buffer. The decalcifying acid buffer was prepared by mixing approximately equal volumes of 0.4 M sodium acetate and 0.4 M acetic acid followed by dropwise addition of 0.4 M sodium acetate until the pH of the solution was 4.70 as indicated on a Cambridge pH meter (reading to ± 0.02 pH units). As this buffer is very nearly 0.2 M with respect to both sodium acetate and acetic acid it will subsequently be referred to as 0.2 M acetic acid buffer, and any dilutions of the buffer will be expressed in terms of this buffer as reference.

Duplicate or triplicate tubes were set up in each instance and gave results agreeing to within 5 per cent of each other. The mean values are presented in the following tables and diagrams.

Rate of decalcification

Decalcification was estimated by analysis of the supernatant solution, for both calcium and phosphate, obtained after shaking the sample in the acid at room temperature. The solution was separated from unreacted solid by centrifugation (4 min, R.C.F. 1500 g), and the supernatant solution either carefully decanted or pipetted from the residue. Preliminary experiments indicated that the decalcification reaction occurred rapidly, there being no further significant decalcification between 1 hr and 48 hr of shaking. In order to ensure equilibrium conditions as far as possible, the samples were shaken for a minimum of 12 hr, generally overnight.

Analytical

Calcium. Estimation of calcium was carried out by precipitation as oxalate followed by titration with permanganate; precipitation of the calcium as oxalate from hot and slightly acid solution prevents the interference from calcium phosphate formation (SCOTT, 1922).

Phosphate. This was estimated by the method of BRIGGS (1924) and the colour measured with a Hilger Biochem Adsorptiometer H.810 (filter 61).

Fluoride. Estimations were carried out using a modification of the method given by the ANALYTICAL METHODS COMMITTEE (1944) and involved distillation of the fluoride with perchloric acid and titration of the fluoride in the distillate against thorium nitrate with alizarin red S as indicator.

Organic matter. The supernatant solutions obtained after acid treatment of the samples tended to be more frothy than distilled water—sample suspensions. This suggested that some organic matter from the samples was present in the supernatant solutions and might possibly interfere with calcium and phosphate analyses. Analysis for calcium and phosphate in the supernatant solutions were the same, whether or not the organic matter had been destroyed by wet ashing with hydrogen peroxide. Hence the organic matter did not interfere with the inorganic analysis.

EXPERIMENTAL

Rate of reaction and equilibrium

In Table 1 are given the calcium and phosphate values in solution after shaking the various samples (500 mg in 100 ml of 0.2 M acetic acid buffer, pH 4.70) for 5 min and for 24 and 48 hr. It can be seen from the results that the extent of decalcification in 5 min, the shortest time at which a result could be obtained, was 90 per cent of that at 48 hr, indicating that under these conditions the reaction was very rapid. In no instance was decalcification complete. In 500 mg of enamel there is approximately 186 mg of calcium and 88 mg of phosphorus, and 135 mg of calcium and 65 mg of phosphorus in the same weight of dentine. This means that 33 and 65 per cent of the normal enamel and dentine respectively were decalcified and the equivalent decalcification for the fluoridated enamel and dentine were 20 and 38 per cent respectively.

In each instance the rise in pH of the buffer solution was small, being roughly proportional to the amount of mineral decalcified.

TABLE 1. MILLIGRAMMES OF Ca AND P LIBERATED FROM 500 mg OF NORMAL AND FLUORIDATED ENAMEL AND DENTINE SHAKEN FOR 5 MIN, 24 HR AND 48 HR RESPECTIVELY IN 100 ml OF 0.2 M ACETIC ACID BUFFER AT ROOM TEMPERATURE

	5 min		24 hr		48 hr		pH at 48 hr
	Ca	P	Ca	P	Ca	P	
Normal enamel	56.7	26.1	62.2	28.1	61.8	28.8	4.80
F enamel (0.43%)	34.3	15.2	39.4	16.5	39.8	16.8	4.72
Normal dentine	83.4	39.8	90.8	41.3	91.4	40.6	4.82
F dentine (0.53%)	45.6	19.3	50.4	21.2	51.1	23.8	4.72

It was thought that perhaps either the tooth mineral had developed a resistance to further acid attack and the reaction was progressing very slowly because of this, or else the buffer solution was saturated with respect to calcium and phosphate. The latter seemed unlikely as more calcium and phosphorus had dissolved from the dentine and the pH of the solution was higher than in the case with enamel.

TABLE 2. MILLIGRAMMES OF Ca AND P LIBERATED FROM 500 mg OF NORMAL DENTINE AND FROM DENTINE CONTAINING 3.7 PER CENT OF F ON SHAKING IN 100 ml OF 0.2 M ACETIC ACID BUFFER, pH 4.70. (1) AND (2) REFER TO ACID OR DENTINE THAT IS COMING INTO CONTACT WITH THE OTHER FOR THE FIRST AND SECOND TIME RESPECTIVELY

	Acid buffer	Dentine	Ca (mg)	P (mg)
Normal dentine	(1)	(1)	91.7	41.6
	(2)	(1)	59.4	21.9
	(1)	(2)	31.6	13.8
F dentine (3.7%)	(1)	(1)	31.0	11.2
	(2)	(1)	14.2	8.6
	(1)	(2)	28.4	10.6

In order to test either of these possibilities the enamel and dentine remaining after treatment with acid were drained by inverting the tubes containing the samples after centrifugation and resuspended and shaken in fresh acid. Similarly, fresh enamel and dentine at the same concentration as before were shaken in acid supernatant solutions that had already once been in contact with the enamel and dentine respectively. In Table 2 are shown the results obtained with normal and fluoridated dentine. Dentine and acid buffer solution which had come into contact with each other for the first time are designated "dentine (1)" and "buffer (1)"; the dentine and acid buffer which had previously been in contact with each other are designated "dentine (2)" and "buffer (2)" respectively. It is obvious from Table 2 that neither had the dentine developed a resistance to the acid solution nor was the solution saturated with respect to calcium and phosphorus.

Decalcification in the presence of excess calcium and phosphate

In Table 3 are shown the results of shaking normal and fluoridated dentine in the acid buffer solution to which had been added different amounts of Ca^{2+} and H_2PO_4^- ions (as CaCl_2 and KH_2PO_4). Decalcification was repressed in the presence of either ion, the decalcification being smaller the greater the concentration of the ion present. This is to be expected for a system in equilibrium.

TABLE 3. MILLIGRAMMES OF Ca AND P LIBERATED FROM 500 mg OF NORMAL DENTINE AND DENTINE CONTAINING 1.2 AND 3.7 PER CENT OF F RESPECTIVELY ON SHAKING IN 100 ml OF 0.2 M ACETIC ACID BUFFER, pH 4.70, CONTAINING PRE-ADDED Ca^{2+} AND H_2PO_4^- IONS

Buffer solution*	Ca (mg)			P (mg)		
	Normal	1.2% F	3.7% F	Normal	1.2% F	3.7% F
A	89.6	35.1	29.8	41.4	16.7	13.1
B	59.9	11.3	7.0	25	5	3
C	76.4	25.5	13.6	38.3	11.5	10.2
D	32	1	1	19.4	2.3	0.87
E	56	20	6	24.7	4.2	1.20

* Buffer solution: A—buffer only
 B—buffer, 149 mg P as KH_2PO_4
 C—buffer, 30.3 mg P as KH_2PO_4
 D—buffer, 354 mg Ca as CaCl_2
 E—buffer, 152 mg Ca as CaCl_2 .

Decalcification at different solid:solution ratios

Amounts of normal and fluoridated enamel and dentine ranging from 50 to 200 mg were shaken overnight in volumes of buffer so as to produce suspension concentrations ranging from 250 to 2000 mg/100 ml of buffer. The solutions were then centrifuged and the supernatant solutions saved for analysis. The results, expressed in terms of 100 ml of buffer, for normal enamel and dentine and for dentine after various fluoride pretreatments are shown in graphical form in Figs. 2 and 3. It is apparent that the solubility in acid is a function of the solid:solution ratio. In each case the pH change in the solution was small and approximately proportional to the amount of material decalcified, the highest pH being reached in the solution initially containing 2000 mg of dentine and even in this instance the pH was only 4.90. The fluoridated samples were less soluble than their counterparts that contained less fluoride and the dependence of their solubility on solid:solution ratio decreased as their fluoride content increased.

On repeated treatment of the sample with fresh buffer solution, decalcification occurred to a smaller extent in each successive case tending towards a different limiting value with each particular sample. For example, the calcium liberated on seven successive treatments of acid from an initial 2000 mg of normal enamel was 114.8, 70.7, 62.9, 57.3, 52.8, 50.9 and 49.5 mg, and from 2000 mg of enamel initially containing 0.43 per cent F, was 42.8, 30.1, 29.2, 29.3, 30.6, 31.2 and 26.4 mg respectively.

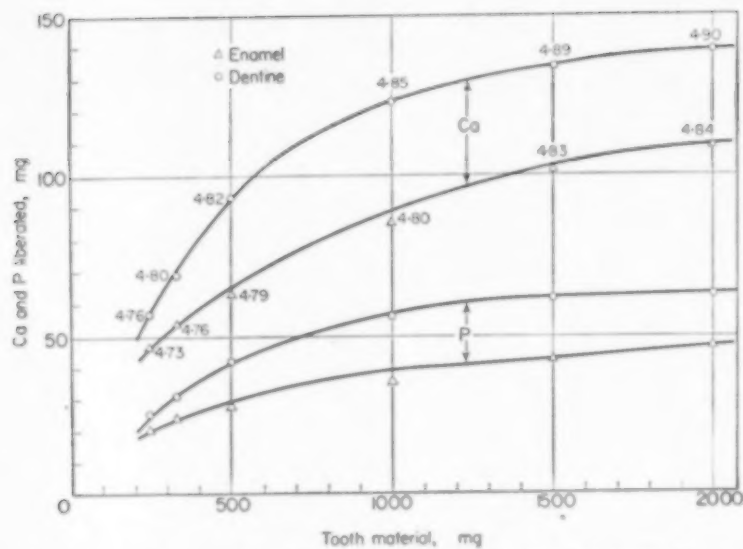


FIG. 2. Milligrammes of Ca and P liberated from different amounts of enamel and dentine shaken in 100 ml of 0.2 M acetic acid buffer of initial pH 4.70. The final pH of the solutions are indicated in the upper portion of the diagram alongside the values of the Ca released.

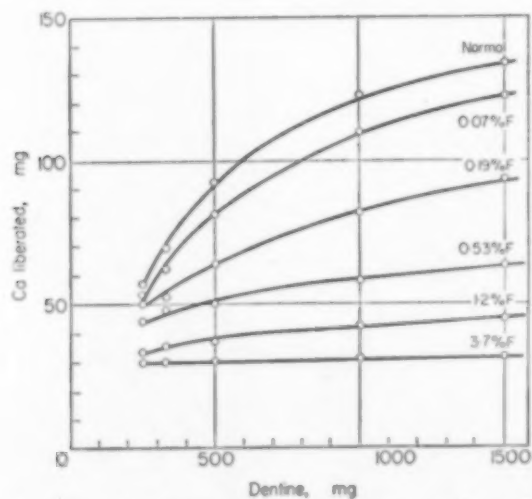


FIG. 3. Milligrammes of Ca released by different amounts of normal dentine and by dentine containing various amounts of fluoride on shaking in 100 ml of 0.2 M acetic acid buffer of pH 4.70.

Decalcification with different strengths of buffer

The effect of diluting the buffer solution and thus reducing its buffering capacity was shown by shaking the samples in 0.2 M acetic acid buffer that had been diluted with distilled water and to which sodium chloride had been added to maintain the same ionic strength. Fig. 4 shows the relationship between the pH of the supernatant solutions after contact with the enamel and dentine at each buffer concentration (expressed in terms of the standard 0.2 M acetic acid buffer) and Fig. 5 shows the calcium liberated at each buffer concentration. In this instance the weaker the buffer solution, the smaller the decalcification, and the higher the rise in pH of the solution. This is to be contrasted with decalcification of different samples with the same buffer strength at a given pH in which the increase in pH of the supernatant solution was a measure of the extent of decalcification (Table 1, Fig. 2).

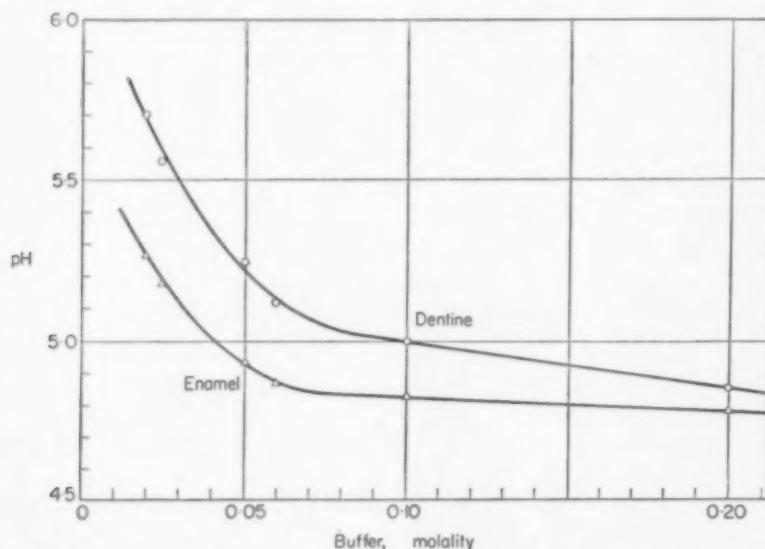


FIG. 4. Final pH of supernatant buffer solutions (100 ml) of different molality (initial pH 4.68 ± 0.02) after shaking with 1 g amounts of enamel and dentine.

Decalcification at different pH values

Buffer solutions with pH values ranging from 3.54 to 5.74 were prepared by mixing together appropriate amounts of 0.4 M acetic acid and 0.4 M sodium acetate solutions. In Table 4 are given the initial and final pH values and the calcium and phosphorus concentrations of supernatant solutions obtained by shaking 1.0 g amounts of normal and fluoridated enamel and dentine in 100 ml of buffer solutions. Each sample was more soluble in the more acid solution. It is to be noted that, even with buffer solutions at a pH of 3.54, decalcification is by no means complete and the

pH does not change to any appreciable extent, i.e. to a pH of 4.12 in the case of normal dentine. Also, at a pH quite near to 6, a significant amount of tooth material still dissolves.

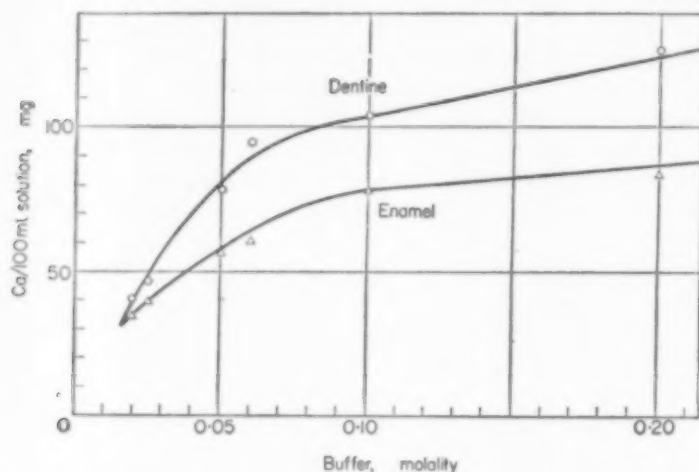


FIG. 5. Milligrammes of Ca released on shaking 1 g amounts of enamel and dentine in 100 ml of buffer solution (initial pH 4.68 \pm 0.02) of different molality.

TABLE 4. THE pH OF Ca AND P (IN MILLIGRAMMES) LIBERATED BY 1 g OF NORMAL AND FLUORIDATED ENAMEL AND DENTINE SHAKEN IN 100 ml OF 0.4 M ACETIC ACID BUFFER AT DIFFERENT pH VALUES

	pH		mg/100 ml	
	Initial	Final	Ca	P
Normal dentine	3.54	4.12	217	98
	4.00	4.34	207	92
	4.70	4.86	126	56
	5.74	6.00	36	14
Normal enamel	3.54	4.00	170	77
	4.00	4.20	132	55
	4.70	4.80	85	35
	5.74	5.86	20	8
F dentine (3.7%)	3.54	3.78	87	37
	4.00	4.08	63	26
	4.70	4.72	32	12
	5.74	5.74	13	4
F enamel (1.8%)	3.54	3.76	67	29
	4.00	4.06	47	21
	4.70	4.72	27	10
	5.74	5.74	12	4

Fluoride in solution

It is possible to differentiate between fluoride released from calcium fluoride and from fluorapatite from samples shaken in distilled water by analysis of the supernatant solution for fluoride and calcium. As calcium fluoride is more soluble than fluorapatite at a neutral or alkaline pH, the calcium and fluoride concentrations in solution are very nearly those expected from calcium fluoride when shaken alone in the solution. Repeated washings of samples containing fluorapatite and calcium fluoride preferentially dissolve the latter because of its greater solubility (LEACH, 1959).

TABLE 5. Ca AND F (IN MILLIGRAMMES) RELEASED FROM VARIOUS FLUORIDATED SAMPLES OF DENTINE (1 g IN 100 ml) AFTER SHAKING IN 0.2 M ACETIC ACID BUFFER (pH 4.70), AFTER FIVE PRIOR SUCCESSIVE WASHINGS IN WATER

	Water		Buffer	
	Ca (mg)	F (mg)	Ca (mg)	F (mg)
F dentine (0.19%)	0.43	0.05	120.0	0.002
F dentine (0.53%)	0.43	0.08	58.0	0.08
F dentine (3.7%)	1.04	1.06	29.8	0.13
F dentine (1.5%) washed free of CaF_2 prior to treatment with acid	0.4	0.05	31.0	0.12

1 mg in 100 ml = 10 parts per million.

In Table 5 is shown the fluoride and calcium released from various fluoridated samples on shaking in distilled water prior to treatment with acid, and the concentration of the same ions released on shaking in 0.2 M acetic acid buffer pH 4.70.

In each instance it is apparent that acid attack and release of calcium depresses the concentration of fluoride in the solution, as expected from the mass law or from solubility product considerations.

DISCUSSION

It seems apparent that the reaction between powdered tooth material and acid rapidly comes to equilibrium. This is certainly not the case for whole teeth, and it is well known that decalcification of whole teeth in acid takes several days. The difference in reaction time must be a result of the relative fraction of the tooth material presented to the acid at any stage in the process and the relative diffusion rates of acid and soluble ions of the mineral to and from the crystal surfaces.

The greater solubility of dentine, and in fact the greater reactivity of dentine than enamel to other reactions such as its ability to take up fluoride, appears to be a result of its smaller crystal size and higher specific surface (WOOD, 1947). If this were not the case it would be expected that enamel would be the more reactive because of its greater inorganic content.

The implication in the dental literature that the pH of an acid solution in contact with tooth mineral should approach neutrality with consequent decalcification is at variance with the results in the present series where but small change in pH and the failure of the enamel and dentine to decalcify have been observed. So also is the implication that the lower the pH of a solution after reacting with tooth mineral then the more calcium and phosphate should be released into solution. It is apparent from the results obtained at different solid:solution ratios with the same acid that this is not the case (Fig. 2). From the results with a given buffer at a fixed initial pH, it is apparent that there is a direct relationship between the rise in pH of the solution and the amount of mineral dissolved (Table 4, Fig. 2). Whereas using acid buffers with identical initial pH values, but of different molal concentrations, the rise in pH is inversely related to the amount of mineral dissolved (Figs. 4 and 5). This means that the pH of a solution when taken as an isolated parameter does not signify a great deal in relation to what has been happening at, say, a tooth surface.

An empirical relationship between the hydrogen ion (or more correctly the hydronium ion H_3O^+) concentration expressed as pH and the concentration of calcium $[\text{Ca}^{2+}]$, in solution at equilibrium with hydroxyapatite, has been given by HODGE (1951) from solubility data collected from the literature. This may be expressed in the form

$$\log[\text{Ca}^{2+}] = K - \text{pH},$$

where K is an empirical constant. The results in the present series in general fit this relationship to the same degree of accuracy as HODGE's collected results when plotted on the graphical representation given by him, even though some of the results in the present series show a direct relationship between $\log[\text{Ca}^{2+}]$ and pH. The increase in solubility of any particular entity in acid solution of lower pH (Table 4) is consistent with the above relationship. It has been tentatively proposed (NEUMAN and NEUMAN, 1958) that there is a direct mole for mole exchange between calcium and hydronium ions at the crystal surfaces of the mineral and hence a direct competition for surface sites between these two ions. Unfortunately it is almost impossible to study pH as an independent variable because any change in pH results in a corresponding change in $[\text{OH}^-]$, $[\text{Ca}^{2+}]$, Ca/P, and $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$. However this proposal provides an explanation for the increased solubility and the greater rise in pH with increase in solid:solution ratio in a given acid solution, the greater reactivity of dentine than enamel in acid because of its larger surface available for exchange and the decreased solubility with concomitant greater rise in pH in solutions of weaker buffering capacity.

The dependence of solubility on solid:solution ratio has also been shown elsewhere within the pH range 7 ± 0.3 (LEVINSKAS and NEUMAN, 1955). In this instance, as well as in the present series, the solubility data could not be made to fit into any constant solubility product relationship, even when the activities rather than the concentrations of the various possible ionic species in solution were considered. This is probably because the activity of the solid phase, and hence the activity of the ions in solution, varies under different conditions.

The solubility of fluoridated mineral, which presumably is fluorapatite, appears to be inversely related to the concentration of fluoride incorporated into the mineral.

It has been shown elsewhere (LEACH, 1956, 1959) that the amount of fluorapatite formed on shaking enamel and dentine with fluoride is in direct relation to the concentration of fluoride in the solution (Fig. 1). Admittedly the majority of the fluoridated powdered tooth samples contain seemingly high, unphysiological concentrations of fluoride compared with whole teeth. However, in view of the reports from two different laboratories (JENKINS and SPEIRS, 1953; BRUDEVOLD, GARDNER and SMITH, 1956) that the surface of the tooth contains more fluoride than the inner portions (in one case, BRUDEVOLD *et al.* (1956), the estimate of fluoride concentration at the surface was 1 per cent), it is possible that there is a significant reduction in the solubility of whole teeth containing a high concentration of fluoride at the surface but present to such a small depth within the tooth that the fluoride content of the tooth taken as a whole is still very small. Because of the large number of other factors involved with whole teeth either *in vivo* or *in vitro* these differences may only be apparent after recourse to statistical analysis: it is apparent even from the results in the present series that there is an appreciable difference in the solubility between normal and fluoridated tooth mineral depending on the condition of the reaction.

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THE OCCURRENCE AND ORIGIN OF CERTAIN VITAMINS IN HUMAN SALIVA*

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Abstract—The chemical forms of certain B-vitamin groups, namely folic acid, pantothenic acid and vitamin B₆, were studied in incubated human saliva by chromatographic procedures. Furthermore, to study the possible contribution of bacteria to vitamin levels in saliva, the population changes of certain bacterial groups were followed. Pure cultures of streptococci, lactobacilli, fusobacteria and veillonellae isolated from saliva were also used to determine whether they would change the vitamin levels in saliva.

The identification of the vitamin forms was made by comparison with known standards on the chromatograms. Chromatography of saliva revealed that compounds similar to folic acid and coenzyme A are present in fresh autoclaved saliva. Upon incubation of fresh saliva, folic acid, citrovorum factor and an unidentified folic acid-like compound, coenzyme A and pyridoxine became detectable.

Bacteriological studies of incubated salivas showed that fusobacteria and veillonellae grew in the absence of added glucose, whereas streptococci and lactobacilli increased only in the presence of added glucose.

Results from pure culture saliva studies indicate that, of the organisms studied, the streptococci were the only ones consistently capable of growth. This was accompanied by an increase in folic acid levels. Vitamin B₆ levels were increased by all pure cultures but the pantothenate levels were variable.

INTRODUCTION

THERE have been several reports on vitamin levels in saliva (KNIESNER, MANN and SPIES, 1942; HILL and KNIESNER, 1942; WEISBERGER, 1946; GLAVIND *et al.* 1948; GRANADOS *et al.* 1950; DREIZEN, REED and SPIES, 1951; KAUFFMAN, KASAI and KOSER, 1953; and NEVIN, APPLEMAN and KURTZ, 1958a) but none dealing with the identity of the chemical forms in which they exist. Furthermore, there is no substantial body of evidence on the origin of the growth factors in this fluid. It is the purpose of this paper to provide information, obtained by chromatographic procedures and microbiological assay, on the identity of those compounds in saliva which have vitamin B₆, pantothenic acid, or folic acid-like activities, and also evidence of their formation by the indigenous oral flora.

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MATERIALS AND METHODS

Saliva. Paraffin-stimulated saliva for the various experiments was collected at mid-morning from seven to ten members of the N.I.D.R. staff. The collected saliva was pooled (400 ml) and kept at 5-0°C until used.

For the purpose of studying the growth of indigenous oral bacteria and changes in vitamin levels in saliva, three aliquots of 50 ml were added to sterile 125 ml Erlenmeyer flasks. One flask, which served as the control, was autoclaved at 121°C for 10 min, the second flask was unaltered and the third flask received 0.63 ml of sterile 40% glucose solution to make the added glucose concentration to 0.5%. These flasks were stoppered with rubber stoppers and incubated at 37°C. At zero time and 20 hr of incubation, samples were removed for bacterial counting. Organisms used in the pure culture studies were isolated from these plates. The contents of the flasks were sterilized by autoclaving at 121°C for 10 min.

For the purpose of studying the growth and vitamin formation of pure cultures, pooled saliva was autoclaved, adjusted aseptically to pH 7, and transferred to 20 × 150 mm sterile test-tubes in 12 ml quantities. To one half of the tubes sterile 40% glucose was added to give 0.5% concentration.

Bacteriology. For the enumeration and isolation of lactobacilli, streptococci, veillonellae and the fusobacteria, pour plates of the following media were employed: SL (ROGOSA, MITCHELL and WISEMAN, 1951), CVI (ROGOSA, SHIOTA and DISRAELY, 1956), V₁₈ (ROGOSA *et al.* 1958), and FM-2 (OMATA and DISRAELY, 1956), respectively. The CVI medium had the following composition: horse-meat infusion containing 0.5% NaCl, 1% trypticase, 0.0002% crystal violet, 0.01% sodium azide, 1.5% agar and 0.5% defibrinated sheep blood.

Pure cultures of lactobacilli, streptococci and fusobacteria were maintained in a fluid thioglycollate medium, and veillonella in V₁₈ medium (without agar) in 16 × 150 mm screw-cap tubes.

The inocula for pure culture studies were prepared by growing the various micro-organisms for 24 hr, washing them twice with sterile water and suspending them in sterile water to an optical density of 0.1 (660 mμ, 18 mm light path, Beckman Model B Spectrophotometer). Tubes containing sterile saliva were inoculated with 0.1 ml of each culture; 3-4 ml was removed for initial bacterial and vitamin determinations, and the rubber stoppered tubes were then incubated for 72 hr at 37°C. After aliquots were removed for bacterial counts, the remaining samples were sterilized by autoclaving at 121°C for 10 min and stored at 5°C until assayed for vitamin content.

Assay. Folic acid-like materials in saliva were measured using *Streptococcus faecalis* R 8043 as assay organism and leucovorin (Lederle) as standard according to the procedure of SILVERMAN and GARDINER (1956) and reported as *S. faecalis* activity. Total folic acid-like activity was measured with *S. faecalis* after treatment of salivary samples with chick-pancreas conjugase (MIMS and LASKOWSKI, 1945).

Pyridoxine-group vitamins were assayed using *Saccharomyces carlsbergensis* 4228 with pyridoxine hydrochloride as standard according to the procedure of ATKIN *et al.* (1943) and reported as *S. carlsbergensis* activity.

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Pantothenate-group vitamins in saliva were measured using *Acetobacter suboxydans* 621 with calcium pantothenate as standard according to the procedure of LANDY and STREIGHTOFF (1943) and reported as *A. suboxydans* activity.

Chromatography. Saliva was centrifuged at $30,000\times g$ for 20 min and the supernate was lyophilized. Fifty per cent ethyl alcohol was added to achieve a tenfold increase in concentration.

Whatman No. 1 paper was used in the development of the folic acid-group chromatograms, and either Whatman No. 1 or the high capacity Whatman No. 17 was used to develop the chromatograms of the vitamin B₆ and pantothenic acid groups. Potassium phosphate, pH 7.0, 0.1 M, was used as the developing solvent for folic acid-like compounds (ZAKRZEWSKI and NICHOL, 1953) water-saturated *n*-butanol for the pyridoxine-group vitamins (WINSTEN and EIGEN, 1948) and *n*-butanol-acetic acid water (250:60:250 V/V/V) (WOIWOOD, 1949) was used for pantothenic acid-like compounds. The papers were developed by the descending method. The chromatograms were air-dried and autoclaved (121°C for 5 min) and bioautograms were prepared by seeding 1.5 per cent agar-supplemented assay media with the proper organisms. In addition to the aforementioned three assay organisms, a fourth, *Lactobacillus arabinosus* 8014, was used to test for pantothenate in a semisynthetic medium (SHIOTA, 1958).

EXPERIMENTAL

Chromatography for vitamins. The effects of incubation on the folic acid-, pantothenate- and vitamin B₆-like activities of autoclaved (control), untreated and glucose-supplemented saliva from three different experiments were studied by chromatographic-bioautographic techniques. Results of typical bioautograms from two or more replicate analyses are presented diagrammatically in Fig. 1.

Folic acid-like activity in samples obtained from control salivas was confined to spots which moved like the folic acid marker. That this activity was not demonstrated in every sample was probably a result of the low levels of the material in such samples, amounting to less than 1 μg of *S. faecalis* activity per millilitre of whole saliva. When samples of incubated salivas were chromatographed, substances with mobilities corresponding to both folic acid and citrovorum factor were detected on the bioautograms. In addition, unidentified materials migrating between folic acid and citrovorum factor were also detected. All of these activities were consistently demonstrated in salivas incubated with added glucose, but when glucose was omitted the substances demonstrating citrovorum factor and folic acid mobilities were not always present.

HAKALA and WELCH (1957) have shown that diapterin (γ -linked pteroyl-L-glutamyl-L-glutamic acid) has an R_F of 0.54, intermediate between citrovorum factor (0.71) and folic acid (0.33), and is active for *S. faecalis*. In one of our analyses the unknown had an R_F value of 0.55, intermediate between folic acid (0.36) and citrovorum factor (0.77).

Vitamin B₆ activity for *S. carlsbergensis* was not detected in samples of control salivas, but was detectable in samples of incubated salivas. The material detected showed a mobility similar to both pyridoxal and pyridoxine (Fig. 1).

A. suboxydans activity was manifested by the presence of two spots on pantothenate chromatograms when incubated saliva samples were used. The first of these spots resembled both pantotheine and coenzyme A, which move only slightly, whereas the second resembled both calcium pantothenate and panthenol in its mobility. Activity, when present in the samples from the control salivas, showed a mobility similar to that of coenzyme A and pantotheine (Fig. 1).

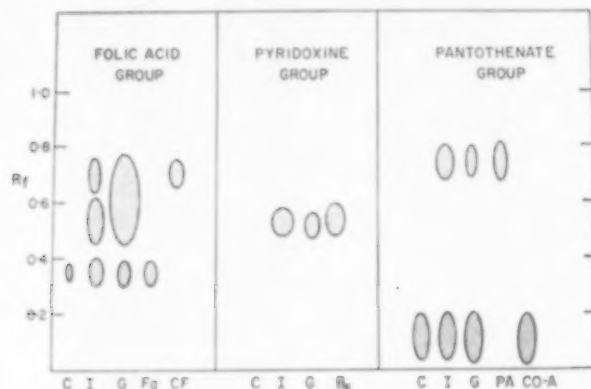


FIG. 1. Chromatography of folic acid-, pyridoxine- and pantothenate-like materials in saliva.

The symbols of the various materials and the amounts used are: Fa, folic acid, 0.5 μ l of 500 μ g/ml; CF, citrovorum factor, 0.5 μ l of 100 m μ g/ml; B₆, pyridoxine hydrochloride, 5 μ l of 5 μ g/ml; PA, calcium pantothenate, 5 μ l of 200 μ g/ml; CO-A, coenzyme A, 10 μ l of 200 μ g/ml; and the following tenfold concentrated saliva preparations were used at 0.1 ml; C, control; I, incubated; and G, incubated with glucose.

Microbiological assay of vitamins. The vitamin activity of the incubated salivas was assayed in aliquots taken from two of the three pooled saliva samples used in the chromatographic studies. The results are presented in Table 1. The activities of the various vitamins in the autoclaved salivas were less than 1 m μ g of *S. faecalis* activity per millilitre for the folic acid group, between 1 and 2 m μ g of *S. carlsbergensis* activity per millilitre for the vitamin B₆ group, and 600–800 m μ g/ml of pantothenic acid activity for *A. suboxydans* in two experiments. After incubation, the level of *A. suboxydans* activity increased almost twofold in the non-supplemented saliva, but no significant change in this activity was found in the 0.5% glucose saliva. The vitamin B₆ group activities in the saliva increased markedly on incubation and this activity was enhanced by the presence of glucose in the incubated saliva. Free folic acid levels were only slightly increased in salivas incubated without glucose, but most markedly increased in the presence of the carbohydrate.

Treatment of the salivas with chick folic acid conjugase revealed amounts of bound folic acid activity for *S. faecalis* in large excess of that freely available. When the total *S. faecalis* activity of incubated salivas was compared to the controls, it was found that in salivas without glucose the increase was twofold or more, and that glucose supplementation gave increases of four- to twenty-fold.

TABLE 1. VITAMIN LEVELS OF INCUBATED SALIVAS

Saliva treatment	<i>S. faecalis</i> activity (m μ g/ml)		<i>S. carlsbergensis</i> activity (m μ g/ml)	<i>A. suboxydans</i> activity (m μ g/ml)
	Free	Total		
Control	0.8	5.04	0.99	785
Incubated	1.1	16.8	5.5	1405
Incubated with glucose	3.62	25.2	11.5	778

Bacterial changes. To show that microbial growth occurred in salivas, two determinations each of *Fusobacterium*, *Streptococcus*, *Lactobacillus* and *Veillonella* populations in incubated salivas were made. These data which are shown in Table 2 are in agreement with those reported earlier by SHIOTA and KUNKEL (1958). *Fusobacteria* and *veillonellae* increased in the absence of added glucose, and streptococci and lactobacilli increased where glucose was present. A slight increase in the numbers of *veillonellae* was also observed where glucose was present.

TABLE 2. BACTERIAL POPULATION CHANGES IN INCUBATED SALIVAS

Organisms	No. of organisms per millilitre of saliva		
	Zero time	Incubated	Incubated with glucose
<i>Fusobacteria</i>	500	1,000,000	10,000
<i>Streptococci</i>	30,000,000	20,000,000	75,000,000
<i>Lactobacilli</i>	68,000	64,000	1,800,000
<i>Veillonellae</i>	210,000	9,300,000	550,000

Pure culture study. Two determinations were made of the ability of certain pure cultures to grow in, and to change, the vitamin levels of autoclaved saliva. Typical population changes of these pure cultures are shown in Table 3. In the presence or absence of added glucose the *Lactobacillus* and *Veillonella* population levels decreased. The streptococci on the other hand showed enormous increases, which were further stimulated by the addition of glucose. The results of *fusobacteria* counts were not consistent, and while those shown suggest increases in their numbers, another experiment demonstrated a decrease. When the four groups of organisms were mixed, only the streptococci showed an increase.

The results of the vitamin assays of salivas inoculated with pure cultures were not always clear and hence are summarized in the text only. Salivas, inoculated with either single pure cultures or a mixture of the twelve strains used in the study, showed

an apparent increase in the level of free folic acid-like compounds. Only the streptococci and the veillonellae cultures showed consistent increases in the total folic acid activity, and this activity was observed mainly in those salivas containing added glucose.

TABLE 3. PURE CULTURE POPULATION CHANGES IN INOCULATED SALIVA

Culture	No.	No. of organisms per millilitre of saliva		
		Before incubation	Incubated	Incubated with glucose
<i>Fusobacterium</i>	1	20	4600	1000
	2	<5	10	<10
	3	27	16,000	46
<i>Streptococcus</i>	10	705	3,000,000	3,800,000
	11	465	2,800,000	8,800,000
	12	430	5,000,000	7,500,000
<i>Veillonella</i>	1	3000	20	20
	3	1800	40	40
	4	<5	30	10
<i>Lactobacillus</i>	2	370,000	500	500
	10	290,000	<500	<500
	22	1,300,000	2250	500
Mixed culture:				
<i>Fusobacteria</i>		<5	<10	<10
<i>Streptococci</i>		350	1,400,000	1,200,000
<i>Veillonellae</i>		90	<10	<10
<i>Lactobacilli</i>		1,300,000	330,000	230,000

Vitamin B₉ activity was increased by pure culture incubation in every instance, and the increase was generally enhanced when glucose was present. The assays for pantothenate-like compounds indicated generally slight increases but were not consistent within any pure culture group studied.

DISCUSSION

The data presented here indicate that certain of the vitamins known to be present in salivas can be formed as the result of microbial action. Such other possible sources contributing to the level of vitamins in saliva, as food ingestion, tissue breakdown or the seepage of fluids from the gingiva, have been considered. TAYLOR, POLLACK and WILLIAMS (1942), and NEVIN, APPLEMAN and KURTZ (1958b) demonstrated vitamins in tissues, and NEVIN *et al.* (1958a) demonstrated that salivary biotin levels increased after food ingestion. However, the relative contributions of the different sources of vitamins remain to be determined.

Autoclaved saliva showed a low level of free folic acid, in agreement with GLAVIND *et al.* (1948) (0.1 m μ g/ml) but far less than that reported by KAUFFMAN

et al. (1953) (24 m μ g/ml). The use by the KAUFFMAN group of a different assay organism may account for the higher result if their organism, a homofermentative oral lactobacillus, is capable of utilizing the conjugated derivatives of folic acid. For example, HAKALA and WELCH (1957) have shown that *Lactobacillus casei* (ATCC 7469) can utilize di- and tri-glutamyl derivatives of folic acid.

Vitamin B₆ activity, probably as pyridoxine or pyridoxal, found in saliva was comparable to that reported by KAUFFMAN *et al.* (1953) (6 m μ g/ml) but was much lower than that reported by GLAVIND *et al.* (1948) (600 m μ g/ml). A possible explanation of GLAVIND's high value lies in the use of *Streptococcus faecium* No. 51 (*S. faecalis*) which requires at least a thousand times more pyridoxine than pyridoxal for equivalent growth. Since GLAVIND used pyridoxine for the standard, salivary pyridoxal would yield very high values. From these data one may infer that salivary vitamin B₆ is chiefly pyridoxine.

GLAVIND *et al.* (1948) using *S. faecium* No. 51, and NEVIN *et al.* (1958a) using *Streptococcus salivarius*, reported similar levels (70–80 m μ g/ml) of pantothenic acid activity in human salivas. The use of *A. suboxydans*, however, an organism capable of utilizing a wide range of pantothenate-active compounds, demonstrated *A. suboxydans* activity levels corresponding to 600–800 m μ g of calcium pantothenate per millilitre of saliva. Since chromatograms indicate that the bulk of this activity is due to compounds moving like pantotheine or coenzyme A, it is probable that pantothenic acid as such constitutes only a small portion of the *A. suboxydans* activity.

Incubation of saliva resulted not only in the increase of free folic acid but also of bound folic acid compounds. Furthermore, chromatography of incubated saliva indicated the presence of an unidentified folic acid-like compound and a compound suggestive of citrovorum factor.

The increase in the *S. faecalis* and *S. carlsbergensis* activity occurred both with and without the addition of glucose to the incubated salivas, but was greater when glucose was present. These data suggest that the lactic acid bacteria, which grew in incubated saliva, could be active in affecting the level of B₆ and folic acid-like compounds in saliva. This does not exclude other organisms from this synthetic role.

The results of the preliminary study of the effect of various pure cultures grown in autoclaved saliva indicate that it is not a suitable environment for growth of the organisms tested, excepting the streptococci. Whether this is due to autoclaving or the omission of a suitable symbiotic system is not known. KOFT and MORRISON (1956) and NURMIKKO (1955) using several known organisms in laboratory media, have successfully demonstrated symbiotic biosynthesis of folic acid-like compounds.

Unlike the folic acid and pyridoxine vitamin groups, the *A. suboxydans* activity is increased in incubated saliva only when glucose is not added to the saliva and, indeed, this activity may be decreased in saliva incubated with added glucose. This increase in the level of *A. suboxydans* activity is suggestively associated with increases in the *Fusobacterium* and *Veillonella* populations, but in the absence of unequivocal supporting data no assumption of a relationship may be made.

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THE INFLUENCE OF SOCIAL AND ECONOMIC CONDITIONS ON THE PREVALENCE OF DENTAL CARIES

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Abstract—A comparative study of dental caries prevalence in children from contrasting social levels was made in order to find whether differences existed between children of differing socio-economic background.

The data were obtained from a stratified sample of 1730 children aged 5-17 years. From this sample 263 children who attended fee-paying schools as day pupils were matched, child for child, in respect of age, sex and number of permanent teeth erupted with an equal number of children who attended non-fee-paying schools.

Dental caries prevalence was measured by the D.M.F. index but, to permit a more extensive comparison, analysis was also made of decayed and filled surfaces.

In the deciduous dentition comparison was confined to the canine and molar teeth of children aged 5-8 years, while in the permanent dentition comparison was made only between children who had erupted from twenty-five to twenty-eight permanent teeth.

The results show that the fee-paying children had a lower prevalence of caries of the deciduous teeth, but a higher prevalence of caries of the permanent teeth.

A comparison of decayed and filled surfaces showed, in general, that for both dentitions caries of smooth surfaces contributed most to the differences in caries between the two classes of children, while the differences attributed to caries of pitted surfaces were generally insignificant.

The findings, interpreted in the light of data from previous studies, suggest that, in the deciduous teeth, susceptibility to caries is influenced more by the quality of the tooth structure, but that in the permanent teeth, factors originating in the oral environment of the teeth exert the greater influence. Possible reasons for this are discussed.

From the evidence obtained it is concluded that significant differences in dental caries experience exist between children from differing social levels and that these differences originate in the total environment of the child. Standards of child welfare constitute the major difference in general environment between the social classes studied and consequently maternal knowledge and efficiency may be more important than economic factors alone.

STANDARDS of diet and nutrition are probably among the more important factors associated with socio-economic conditions which can influence health. They are also important factors in determining susceptibility to dental caries.

In children, social class differences in rates of growth and development have been shown. Over the years, increases in the rates of growth of children have occurred which are thought to reflect the rising standard of living (CLEMENTS, 1953). Social class differences in food consumption have been shown ("Domestic Food Consumption and Expenditure, 1953", 1955) and it was found that the nutrient value of the diets declined from the higher to the lower social groups.

During both World Wars, considerable changes in standards of living occurred, particularly with regard to diet, and these dietary changes are thought to be responsible for the very marked decline in prevalence of dental caries which was seen in European children during and following the wars (SOGNNAES, 1948; TOVERUD, 1956). Clearly, susceptibility to dental caries would seem to be sensitive to variations in

standards of living where these also influence diet; therefore, differences in the prevalence of dental caries could be expected between individuals from differing socio-economic backgrounds. The literature, however, reveals findings which are in conflict in some respects.

Thus, in the permanent dentition, a higher susceptibility to caries in individuals from higher socio-economic levels has been reported (Report of the Interdepartmental Committee on Physical Deterioration, 1904; MITCHELL, 1933; HYDE, 1944), but KLEIN and PALMER (1942) found no evidence that socio-economic factors influenced susceptibility to dental caries.

In the deciduous teeth, however, a lower prevalence of dental caries has been reported in children with higher socio-economic backgrounds (WILKINS, 1941; MELLANBY and COUMOULOS, 1944; COUMOULOS and MELLANBY, 1947). SAVARA and SUHER (1955), on the other hand, found no association between income or occupation of parents and dental caries in their children, but they did find an association between dental caries in children and standards of parental education.

Therefore, in an attempt to resolve these conflicting findings, the present study was undertaken.

METHODS

The data were obtained from part of a sample of 1730 day-school children aged 5-17 years, selected to provide proportional representation of all social classes. The sampling procedure has been described elsewhere (PROVIS and ELLIS, 1955).

The children were divided into two groups: those attending private, fee-paying schools and those attending local-authority schools (non-fee-paying). Thus two groups of children, with contrasting socio-economic backgrounds, were obtained; those who attended the fee-paying schools belonged predominantly to Social Class 1, while those attending the local authority schools represented Social Classes 3, 4 and 5 (Registrar General, 1931).

The number of fee-paying children at each age was relatively small compared with the non-fee-paying children and therefore no attempt was made to compare them by age. Instead two groups were selected from the total sample of fee-paying children, each large enough to permit statistical analysis. The first group contained all children from 5 to 8 years of age, while the second group comprised all children who had erupted not less than twenty-five and not more than twenty-eight permanent teeth.

Two comparable groups of non-fee-paying children were created by matching each fee-paying child with a non-fee-paying child of the same age, sex and number of permanent teeth erupted. Thus, for both social classes two groups were obtained, the first to compare caries of the deciduous teeth between the fee-paying and non-fee-paying children, the second to make a similar comparison of the permanent teeth.

Tables 1 and 1(A) show the structure of these groups in respect of numbers of children, age and sex. For the permanent teeth, the average number of teeth erupted per child is also given, but for the deciduous teeth it has been assumed that all teeth had erupted.

TABLE 1. MEAN AGE OF MATCHED FEE-PAYING AND NON-FEE-PAYING CHILDREN IN THE AGE GROUP 5 TO 8 YEARS

	Boys		Girls		Both sexes	
	Fee-paying	Non-fee-paying	Fee-paying	Non-fee-paying	Fee-paying	Non-fee-paying
No. of children	36	36	43	43	79	79
Mean age (years)	7.11	7.08	7.06	7.08	7.08	7.08
Standard deviation (years)	(1.19)	(1.18)	(1.18)	(1.17)	(1.18)	(1.17)

TABLE 1 (A). MEAN AGE AND NUMBER OF PERMANENT TEETH ERUPTED IN MATCHED FEE-PAYING AND NON-FEE-PAYING CHILDREN WHO HAD 25 TO 28 PERMANENT TEETH ERUPTED

	Boys		Girls		Both sexes	
	Fee-paying	Non-fee-paying	Fee-paying	Non-fee-paying	Fee-paying	Non-fee-paying
No. of children	87	87	97	97	184	184
Mean age (years)	15.21	15.21	15.22	15.24	15.22	15.22
Standard deviation (years)	(1.62)	(1.62)	(1.83)	(1.84)	(1.73)	(1.73)
Mean No. of teeth erupted	27.60	27.60	27.61	27.61	27.60	27.60
Standard deviation (teeth)	(0.83)	(0.83)	(0.85)	(0.85)	(0.88)	(0.88)

Dental examination

The children were examined in the schools during school hours and the examinations made using plane mouth mirrors and Ash No. 54 probes. Illumination was obtained by the use throughout of a portable lamp, using a 60 W bulb.

Dental caries and all fillings were recorded for each tooth and for each surface of the tooth involved. All missing teeth were recorded, together with any relevant dental history. Unerupted teeth were recorded as such.

Oral hygiene

Standards of oral hygiene were assessed as "good", "fair" or "neglected", and these in effect formed a continuous scale. The categories were defined thus:

Good. When the teeth were clean and free from staining.

Neglected. When food debris (not of recent origin), materia alba and/or staining of accessible tooth surfaces were found.

Fair. When the condition of teeth was judged to be intermediate between the two extremes.

The measurement of dental caries

The D.M.F. index (KLEIN, PALMER and KNUTSON, 1938) has been used. For the deciduous teeth D.M.F. values were calculated for the molar and canine teeth only.

An analysis of decayed and filled tooth surfaces was also made, in which the various surfaces were classified as "smooth" and "pitted" surfaces, thus:

	Smooth	Pitted
Incisors	Mesial Distal Labial	Lingual
Premolars	Mesial Distal Buccal Lingual	Occlusal
Molars	Mesial Distal	Occlusal Buccal Lingual

Because of the low incidence of caries of mandibular incisors and premolars, insufficient information was available regarding carious surfaces of these teeth and they were therefore excluded from the analysis of tooth surfaces.

By including all lesions and fillings in buccal and lingual surfaces of molars as "pitted" surface lesions, it was recognized that there may be included a proportion of lesions which originated in the gingival third of these surfaces and should therefore be regarded as "smooth" surface lesions. However, in children this class of lesion is relatively infrequent and therefore any error so entailed was thought to be small.

Definition of carious surfaces

Carious lesions and fillings were regarded equally as evidence of carious attack and, where these involved a combination of approximal and occlusal surfaces, they were recorded as caries of the proximal surface only. The occlusal extension was ignored on the grounds of clinical experience that the occlusal surface was most probably secondarily involved as a consequence of caries of the approximal surface or, in the case of a filling, by mechanical extension during cavity preparation. Discrete occlusal lesions or fillings, not in communication with the occlusal extension, were, however, recorded as a separate occlusal attack. A comparison of comparable treated and untreated teeth suggested that this method of recording would produce insignificant error. In those instances where extensive caries or large compound fillings made it impossible to judge the surfaces primarily involved, the total surfaces were recorded in the category "unclassified".

RESULTS

Data are presented to describe and compare dental caries in children from differing social classes. The main comparisons were made as follows:

(a) To provide an estimate of the level of caries in each social group; the mean number of decayed, missing and filled teeth per child was calculated.

(b) To find if differences existed between the social classes in terms of decayed and filled tooth surfaces; the proportions of smooth and pitted surfaces attacked in the various teeth were compared.

As described before, each fee-paying child was matched with a non-fee-paying child. Thus the children studied constituted in effect a series of pairs, in which each pair member was derived from a different social class.

For each pair the difference in D.M.F. teeth between the fee-paying and the non-fee-paying child could be obtained and therefore for all the pairs the mean difference could be calculated.

The mean difference between the pairs was, of course, identical with the difference in D.M.F. teeth between the two groups. Nevertheless, variability measured between the matched pairs was, as a consequence of matching, less than that found for the same children treated as separate groups.

Thus, for the deciduous teeth, the standard error of the difference between the mean D.M.F. of each social group was 0.55, while the standard error of the mean difference between the matched pairs was 0.49.

Similarly, for the permanent teeth, the corresponding standard errors were: for the difference between the mean of the groups, 0.45 and for the mean difference between the pairs, 0.42.

Therefore, the significance of the differences in D.M.F. teeth between the two social classes was estimated from the difference between the matched pairs, because their lower variability gave a more precise estimate of statistical significance.

TABLE 2. DENTAL CARIES OF DECIDUOUS AND PERMANENT TEETH IN FEE-PAYING AND NON-FEE-PAYING CHILDREN MATCHED IN RESPECT OF AGE, SEX AND NUMBER OF TEETH ERUPTED

	Deciduous teeth		Permanent teeth	
	Fee-paying	Non-fee-paying	Fee-paying	Non-fee-paying
No. of children	79	79	184	184
Mean D.M.F. teeth per child	3.90	6.18	7.64	6.67
(\pm Standard error)	(± 0.40)	(± 0.38)	(± 0.35)	(± 0.29)
Mean difference in D.M.F. teeth between paired fee-paying and non-fee-paying children	2.28		0.97	
(\pm Standard error)	(± 0.49)		(± 0.42)	

The summarized data are presented in Table 2 to show the average number of decayed, missing and filled teeth per child and the mean of the difference between the matched pairs of fee-paying and non-fee-paying children.

Deciduous teeth. It can be seen from Table 2 that the incidence of dental caries of deciduous molar and canine teeth is greater in the non-fee-paying children. The difference in caries between the two classes of children is statistically highly significant and the probability that such a difference could occur by chance is less than 1 in 1000 ($t=4.67$, $p<0.001$).

Permanent teeth. In contrast to the deciduous teeth, the differences between the two classes of children in regard to caries of the permanent teeth are reversed; the fee-paying children show the *greater* number of decayed, missing and filled teeth per child.

The mean difference between the matched pairs of children is statistically significant at the 2 per cent level. The probability that such a difference could be found by chance is approximately 1 in 50 ($t=2.32$, $p=0.02$).

Distribution of decayed and filled surfaces

Deciduous teeth. From Table 3 it can be seen that of the total surfaces examined, the fee-paying children had consistently a smaller proportion attacked.

TABLE 3. PROPORTION OF DECAYED AND FILLED SURFACES OF DECIDUOUS CANINE AND MOLAR TEETH IN MATCHED FEE-PAYING AND NON-FEE-PAYING CHILDREN.

DECIDUOUS TEETH							
Tooth	Group	No. of teeth examined	Smooth surfaces, percentage decayed or filled	Pitted surfaces, percentage decayed or filled	Unclassified as percentage of total surfaces decayed or filled	Total surfaces, percentage decayed or filled	Missing teeth, as percentage of total teeth erupted
Maxilla Canine	Fee-paying	153	3.7	—	1.6	4.4	3.2
	Non-fee-paying	157	7.9	—	—	5.9	0.6
First Molar	Fee-paying	131	9.9	2.3	—	6.9	17.1
	Non-fee-paying	120	19.7	3.3	—	13.2	24.0
Second Molar	Fee-paying	146	9.6	9.6	—	9.6	7.6
	Non-fee-paying	139	13.9	15.8	0.7	15.4	12.0
Mandible Canine	Fee-paying	100	1.6	—	0.8	2.0	5.1
	Non-fee-paying	148	4.7	—	—	3.5	6.3
First Molar	Fee-paying	136	9.3	1.8	0.4	6.8	13.9
	Non-fee-paying	100	19.3	1.5	0.2	12.4	36.7
Second Molar	Fee-paying	140	7.6	12.1	1.4	10.9	11.4
	Non-fee-paying	108	16.4	7.9	1.7	16.6	31.6

The differences between the two groups of children are illustrated in Fig. 1. They are statistically significant for the molars but not for the canines.

When smooth and pitted surfaces were compared, the fee-paying children were found to have a significantly lower proportion of smooth surfaces attacked for all the teeth examined. However, for pitted surfaces the differences between the two

groups of children were insignificant except for the maxillary second molar, and moreover were inconsistent in trend, as the fee-paying children showed higher rates for the mandibular molars and lower rates for the maxillary.

Permanent teeth. The distribution of decayed and filled permanent tooth surfaces are presented in Table 4 and show that, in contrast to the deciduous teeth, the fee-paying children had a consistently higher proportion of attacked surfaces than the non-fee-paying.

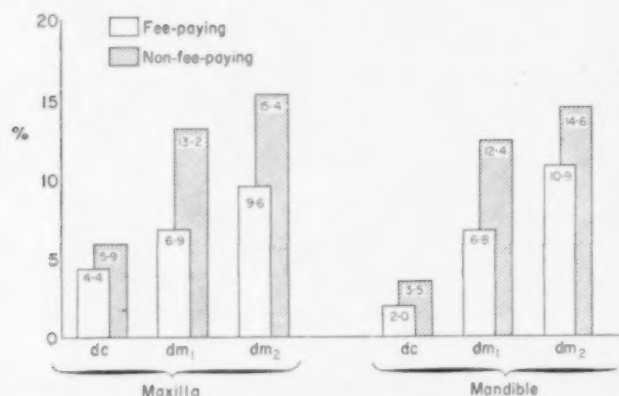


FIG. 1. The distribution of decayed and filled surfaces of deciduous canine and molar teeth in fee-paying and non-fee-paying children, matched for age and sex.

TABLE 4. PROPORTIONS OF DECAYED AND FILLED SURFACES IN PERMANENT TEETH IN MATCHED FEE-PAYING AND NON-FEE-PAYING CHILDREN.

PERMANENT TEETH							
Tooth	Group	No. of teeth examined	Smooth surfaces, percentage decayed or filled	Pitted surfaces, percentage decayed or filled	Unclassified as percentage of total surfaces decayed or filled	Total surfaces, percentage decayed or filled	Missing teeth as percentage of total teeth erupted
Maxilla	Central Incisor	Fee-paying 367	9.7	1.1	—	7.6	0.3
	Non-fee-paying	361	7.5	0.3	—	5.7	1.9
	Lateral Incisor	Fee-paying 366	5.2	5.5	—	5.3	—
	Non-fee-paying	354	3.9	2.5	—	3.6	3.3
	First Premolar	Fee-paying 357	2.6	7.3	—	3.5	1.4
	Non-fee-paying	348	1.2	6.6	—	2.3	3.1
Mandible	Second Premolar	Fee-paying 351	3.7	4.8	—	3.9	1.7
	Non-fee-paying	351	2.8	5.4	—	5.3	2.5
	First Molar	Fee-paying 310	13.5	24.9	0.8	21.2	15.8
	Non-fee-paying	293	8.0	20.2	1.5	16.9	20.4
	Second Molar	Fee-paying 348	2.4	18.3	0.6	12.5	—
	Non-fee-paying	350	0.6	17.2	0.4	11.0	0.3
Mandible	First Molar	Fee-paying 294	17.0	24.0	1.8	23.7	20.1
	Non-fee-paying	253	6.5	20.0	1.7	16.3	31.2
	Second Molar	Fee-paying 356	4.3	19.8	0.3	13.9	0.8
	Non-fee-paying	339	0.4	15.4	0.5	9.9	5.2

The differences between the groups are statistically significant at the 5 per cent level of significance, for all teeth except the maxillary second premolar and second molar. These differences are illustrated in Fig. 2.

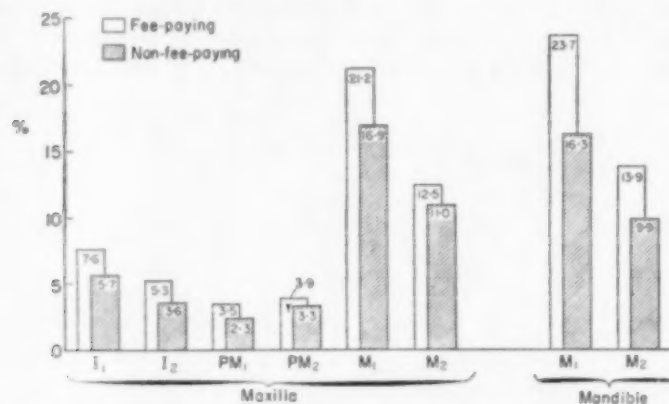


FIG. 2. The distribution of decayed and filled surfaces of various permanent teeth in fee-paying and non-fee-paying children, matched for age, sex and number of teeth erupted.

When the proportions of attacked surfaces were compared for the two groups of children, the fee-paying children showed a consistently higher rate of smooth surfaces attacked for all the teeth examined. The differences are statistically significant for all molars and the maxillary first premolar.

For pitted surfaces, however, the differences between the groups are smaller, although the fee-paying children still show higher rates for all teeth except the maxillary second premolar. The differences in attack of pitted surfaces are only statistically significant for the mandibular second molar, maxillary first molar and lateral incisor.

Standards of oral hygiene

The findings regarding the standard of hygiene found in these children are given in Table 5.

TABLE 5. PROPORTIONS OF CHILDREN IN FEE-PAYING AND NON-FEE-PAYING SCHOOLS WHOSE STANDARDS OF HYGIENE ARE CLASSED AS GOOD, FAIR AND NEGLECTED

	Good (%)	Fair (%)	Neglected (%)
Age 5-00-8-99 years			
Fee-paying	88.6	11.4	—
Non-fee-paying	57.0	40.5	2.5
Aged 15 years			
Fee-paying	72.3	26.1	1.6
Non-fee-paying	54.3	32.6	13.1

It can be seen that differences in the standards of hygiene between fee-paying and non-fee-paying children are considerable and in the direction that would be expected.

Differences in tooth loss

In recording decayed and filled surfaces, missing teeth had to be excluded because no reliable estimate could be made of their attacked surfaces.

The fee-paying children had a lower rate of both deciduous and permanent teeth lost than the non-fee-paying children, consequently they had a greater proportion of tooth surfaces remaining at risk of attack. The possibility that this might have influenced the differences in attacked surfaces found between the two social classes, therefore, had to be considered.

The data presented show, however, that the differences between the children due to this cause are negligible for the following reasons.

The reversal in trend of caries between the deciduous and permanent teeth was seen in both D.M.F. teeth and also decayed and filled surfaces (cf. Figs. 1 and 2 and Table 2).

Thus, the D.M.F. index, which includes missing teeth, can be seen to agree in direction of trend with the findings for attacked surfaces in both dentitions and both social groups. It must be concluded, therefore, that the differences in missing teeth between the two groups have made no material contribution to the differences in attacked tooth surfaces in this study.

DISCUSSION

The results obtained reveal significant differences in the prevalence of dental caries between children from contrasting social groups; namely that the fee-paying children show a lower prevalence of caries of the deciduous teeth, but a higher prevalence of caries of the permanent teeth.

These findings are in agreement with those of WILKINS (1941), MELLANBY and COUMOULOS (1944) and COUMOULOS and MELLANBY (1947) for the deciduous teeth, while for the permanent teeth they agree with the findings of MITCHELL (1933), HYDE (1944) and also with the evidence reported by the Interdepartmental Committee on Physical Deterioration (1904), which stated that in Edinburgh school children a higher rate of defective permanent teeth was found in children of the professional classes compared with working class children.

A comparison of decayed and filled surfaces between the fee-paying and the non-fee-paying children showed that the fee-paying children had a *smaller* proportion of smooth surfaces attacked in their deciduous teeth, but a *higher* proportion attacked in their permanent teeth. On the other hand, the proportion of pitted surfaces attacked was substantially the same in both classes of children. It can be seen, therefore, that the trends in prevalence of caries are reflected in the proportions of smooth surfaces attacked in both dentitions.

There is evidence to suggest that caries of smooth surfaces is more dependent than caries of pitted surfaces upon post-eruptive factors influencing the oral environment of the tooth (REID and GRAINGER, 1955). Thus it is thought possible that the

difference in caries between these children may be due to variations in caries-provoking factors in the oral environments of their teeth.

It is well known that the refined carbohydrate content of the diet constitutes an important environmental factor which may influence susceptibility in caries (GUSTAFSSON *et al.*, 1954). Moreover, social class differences in food consumption have been reported ("Domestic Food Consumption and Expenditure, 1953", 1955), showing the consumption of sugars, preserves, biscuits and other cereals to be greater in the higher than in the lower social groups. Such differences in diet could reasonably explain the greater prevalence of caries of the permanent teeth in the fee-paying children, but would not explain the lower prevalence of caries of their deciduous teeth, found in this and in other studies.

However, susceptibility to dental caries may also be affected by factors which operate during the period of tooth development to influence the resistance of the tooth to attack, thus the environment of the infant in the first year of life in terms of health and nutrition is thought to be of importance in influencing the structural quality of the deciduous teeth (TOVERUD, 1949). Moreover, it has been shown that in most instances pre-natal enamel and dentine appear to be better calcified than that formed post-natally (SCHOUR, 1936). This is supported by the findings of COUMOULOS and MELLANBY (1947) relating the surface structure of enamel to the incidence of dental caries, which suggest that the enamel formed post-natally may be the more important since this is the outer enamel.

Social class differences in infant health are well known (DOUGLAS, 1951), while the rate of mortality in the first year of life shows a steady increase between Class 1 (professional class) and Class 5 (the unskilled labourer) (Registrar-General for Scotland, 1954). COUMOULOS and MELLANBY (1947) found in 5 year old London children that those attending private (fee-paying) schools had teeth of better structural quality than children attending local authority schools. They suggested that the lower caries rate of the private school children was due mainly to greater resistance of their teeth to attack. Furthermore, they found that, within any one social group, those with the poorest enamel structure showed the highest prevalence of caries.

SAVARA and SUHER (1955) found in pre-school children that the prevalence of caries declined as the standards of parental education rose. On the other hand, they found no association between the prevalence of caries in children and either the income of father, the consumption of refined carbohydrates, the frequency of eating between meals or the frequency of tooth-brushing.

It is to be noted that the average age of the children studied by SAVARA and SUHER was 3.9 years and they were therefore considerably younger than those studied by COUMOULOS and MELLANBY and also those in the Edinburgh study. In consequence, the type of lesion predominating at this age is that which originates in pit and fissure systems and has its onset soon after the tooth erupts. This type of lesion may be influenced more by conditions existing during tooth development. On the other hand, smooth surface lesions which are more influenced by factors in the oral environment appear to require a longer duration of exposure to produce this characteristic effect (REID and GRAINGER, 1955). In this event, the negative correlation between

standards of parental education and caries prevalence in the child may be a measure of standards of infant welfare.

These combined findings suggest that, in the deciduous teeth, oral environmental factors in caries production play a subordinate role to factors influencing resistance of the teeth.

Resistance of the tooth to attack does not change appreciably once the tooth enamel has completed formation. Therefore, with the passage of time, environmental effects must tend progressively by accumulation to obscure the effects of resistance.

In the present study, the average age of the children in whom the deciduous teeth were studied was 7 years, while those in whom the permanent teeth were studied were 15 years of age. Thus, the duration of exposure of the permanent teeth to the oral environment was appreciably greater than that of the deciduous teeth.

The effect of length of oral exposure on the proportions of smooth and pitted surfaces attacked by caries is illustrated in Table 6.

TABLE 6. THE PROPORTIONS OF DECAYED AND FILLED SURFACES OF MAXILLARY PERMANENT FIRST AND SECOND MOLARS IN 15 YEAR OLD CHILDREN (Derived from Table 4)

Permanent teeth	Approximate duration of exposure in the mouth	Tooth surfaces decayed or filled	
		Smooth surfaces (%)	Pitted surfaces (%)
Fee-paying			
Maxillary first molar	9 years	13.5	24.9
Maxillary second molar	3 years	2.4	18.3
Non-fee-paying			
Maxillary first molar	9 years	8.0	20.2
Maxillary second molar	3 years	0.6	17.2

From Table 6 it can be seen that, between the first and second permanent molars, the differences in smooth surfaces attacked are roughly proportional to their length of exposure in the mouth. On the other hand, the differences in attacked pitted surfaces are relatively small and appear to be less influenced by duration of exposure. Thus, in the permanent teeth, oral environmental factors could be expected to make the greater apparent contribution to the social class difference in caries, while in the deciduous teeth, with their shorter oral exposure, the effects of resistance to attack by caries have not yet been obscured by environmental effects.

It is worthy of note that the differences in caries between the social classes are very much greater in the deciduous than in the permanent teeth (cf. Table 2) and support the view that standards of child welfare constitute the major environmental difference between the two social classes.

The findings relating to standards of oral hygiene show that the fee-paying children have a much higher standard than the non-fee-paying children. However, the data provide no consistent evidence to show whether standards of oral hygiene had in any way influenced the incidence of caries in these children.

The evidence provided by this study, together with the findings of earlier investigations, permits the conclusion that significant differences in caries experience exist between children from contrasting social levels.

The data obtained in the present study suggest that the lower prevalence of caries of deciduous teeth in the fee-paying children is a reflection of their superior nutrition and health during the first year of life compared with the non-fee-paying children. Clearly, standards of child care contribute to this result and suggest that standards of maternal knowledge and efficiency may be more important than economic factors alone.

On the other hand, the greater prevalence of caries of the permanent teeth in the fee-paying children appears to be related to diet following the eruption of their teeth.

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PROGRESSIVE FACIAL HEMIATROPHY FOLLOWING CERVICAL SYMPATHECTOMY IN THE RAT

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Abstract—Unilateral cervical sympathectomy of the month-old rat produced a condition simulating progressive facial hemiatrophy during a postoperative period of 2-4 months. While the gross extent of the lesion produced varied, the histological appearance was uniform: adipose tissue alone was diminished. Osseous, muscular and vascular structures of the facial skull appeared unaltered under these experimental conditions. No evidence of a neurotrophic influence on either the growth or maintenance of bone form was found. The mechanism of production of the adipose atrophy remains unknown.

INTRODUCTION

PROGRESSIVE facial hemiatrophy in man (Romberg's disease) is characterised by a gross diminution of facial form variably including the skin, subcutaneous fat and connective tissues, with a possible extension to the muscles and bones. Typically, it may begin at any age and in any part of the face and is usually but not necessarily limited to that region. Apart from these broad statements, a survey of some 500 cases (ARCHAUBAULT and FROMM, 1932; DECHAUME, CAUHEPE and DUBRUILLE, 1954) reveals a considerable confusion concerning aetiology, pathogenesis and associated symptoms. In this plethora of data several comments appear with such frequency that interest is focused upon them: (1) facial trauma, including tooth extraction, has often been cited as a primary aetiological factor. Genetic factors appear to be without significance. (2) Atrophy of the underlying bones is said to occur if the onset of the condition is prior to cessation of splanchnocranial growth, despite the fact that the related muscles would show no neurological signs of degeneration. (3) The site of the lesion is usually restricted to the trigeminal area and some direct involvement of this cranial nerve is claimed in many cases.

Quite apart from the obvious maxillo-facial implications of this problem to the several clinical specialities, the suggestion that "trophic" nerves are in any way concerned with either the growth of bone or its maintenance was deemed worthy of investigation. In addition, the authors could find no published reports of animal experimentation on facial hemiatrophy.

MATERIALS AND METHODS

Unilateral (left) sympathectomy, by interruption of the cervical sympathetic chain, was attempted in 23 Long-Evans rats at one month of age. At this age neurocranial growth is virtually completed and any effect of this operation would be

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reflected on the rapidly growing splanchnocranium alone (MOSS, 1954; BAER, 1954; MOSS and BAER, 1956). Sacrifice occurred 2-4 months postoperatively. Twelve rats of similar age served as unoperated controls and 4 additional rats were subjected to sham operations.

The cervical sympathetic chain is closely related to the dorsal aspect of the common carotid artery and to the vagus nerve. This region was reached by a mid-line incision in the neck, an upward reflection of the associated salivary glands and the separation of the infrahyoid muscles. The nodular masses representing the several sympathetic cervical ganglia were sought for to aid in the identification of this nerve. The suspected nerve chain was cut and several millimetres of its length removed (see GREENE, 1955, for full anatomical details). The difficulty of this operation in our hands was reflected by the fact that it was successful in only 14 of the 23 rats. In these there was an immediate and persistent ptosis of the lid on the operated side (Fig. 1).

At sacrifice the rats were decapitated and the heads arranged for photography in such a fashion that the vertical axis of the lens passed to the mid-sagittal line just behind the orbits. The heads were moistened and the hair smoothed back close to the skin. Care was exercised to avoid lateral tilting of the head. From these photographs tracings were prepared in the following manner. The mid-sagittal plane was easily located by a line connecting the half-way points between three anatomical landmarks: the anterior (inner) eye angles, the posterior (outer) eye angles and the anterior (medial) root of the ear (Fig. 2). In all cases the mid-sagittal line and the outline of the right (unoperated) side of the head were traced on overlay paper from the ear to the point where the mid-sagittal line crossed the snout. The tracing paper was then reversed and re-registered, and the left (operated) side traced.

In 12 of the successfully sympathectomized animals blocks of soft tissue were removed from identical facial areas on the operated and unoperated sides for histological examination. Formalin fixation was followed by sectioning at 10 μ and staining with haematoxylin and eosin. The remaining two sympathectomized rat heads which showed the most marked gross changes were treated by boiling in water and bleaching in 3% hydrogen peroxide in order to study gross skeletal changes. Bilaterally similar areas of the several facial bones were also decalcified, sectioned and stained.

RESULTS

Gross observations

Progressive facial hemiatrophy uniformly followed unilateral cervical sympathectomy in all 14 rats. The rate of this process was not determined. An apparent sinking-in of facial contour was observed. The extent of this process was variable, ranging from a barely noticeable, lateral depression to a severe wasting that caused marked deviation of the snout to the side of the lesion. Neither depilation nor stimulation of hair growth was noted, nor were there corneal disturbances. The animals ate well and grew normally. Tooth growth and eruption were bilaterally normal, as was the state of the mucous membranes.

The skulls of the 2 animals with the most marked external alteration of facial contour did not reveal any significant dimensional bony changes in either the neuro- or splanchno-cranium. This was somewhat surprising at first sight, because of the marked deviation of the snout. Despite this, the mid-nasal suture formed a straight line with the metopic and sagittal suture and the premaxillary, nasal and maxillary bones were symmetrical.

Tracings of the photographs of the control rats showed a slight degree of facial asymmetry. Distinct from these minor morphologic variations was the uniform atrophy demonstrated on the operated side in the sympathectomized animals (Figs. 2 and Fig. 3).

Histological observations

The most constant change in the experimental animals was a decrease in the amount of subcutaneous fat (Fig. 4). Quantitatively there was approximately a 60% reduction of this tissue between the dermis and the underlying facial musculature in regions of heavy fat concentration, such as the mystacial pad along the upper lip into which the vibrissae are inserted. The fat loss was virtually total in other, less well-endowed facial regions.

Both the epithelium and the muscle tissue appeared normal. The dermal fibrous connective tissues were unaltered and no vascular changes were observed. Glandular tissue was similarly unaltered.

No osseous changes were observed microscopically. Bone on the sympathectomized side showed no evidence of resorption.

DISCUSSION

These data support belief that unilateral cervical sympathectomy of the young rat produced a condition simulating human progressive facial hemiatrophy. Under the specific conditions of this experiment, loss of subcutaneous adipose tissue was the only change observed.

The similarity between this experimentally produced progressive facial hemiatrophy and Romberg's disease does not necessarily mean that the two conditions are closely analogous. Different aetiologic factors and different pathogenic processes may produce similar symptom complexes. The widely accepted working hypothesis that the sympathetic division of the autonomic nervous system is implicated in Romberg's disease is, however, partially strengthened by the present data. This statement does not exclude other non-sympathetic aetiologies, nor is it claimed that disruption of the sympathetic pathways is required to produce hemiatrophy in all cases.

A more thorough review of the clinical status of this disease, together with a method of analysis which permits distinction between congenital and acquired pathogenic factors will be published elsewhere. Suffice it to say that nothing has been found in our series of cases to invalidate the proposed aetiologic role of the sympathetic nervous system.

"Trophic" nerves

The relationship between the pathogenesis of the produced hemiatrophy and the possible "trophic" role of the cranial sympathetic nerves must be considered. In particular, we are concerned with the role of nerve trophism in growth and maintenance of osseous and connective tissues. Trophism as a concept has a varied history. A recent summary has been given by WYBURN-MASON (1950) who claims an all-encompassing role for trophic nerves. There is no question about the functional relationship between the sympathetic nerves and the vasomotor, pilomotor and sudomotor functions of the skin. There is considerable doubt concerning the direct role of these fibres in either the growth or maintenance of these soft tissues.

Concerning bone the situation is not appreciably clearer. Two conflicting theories have emerged in this regard which attempt to explain the frequently paradoxical data obtained by different workers. The first theory claims that sympathectomy should produce at least a temporary vasodilatation and that this, in turn, should stimulate bone growth (GULLICKSON, KUBICK and KOTTKE, 1951). The second theory claims a more direct primary "trophic" influence on bone and holds that for this reason sympathectomy should decrease the rate of growth (LERICHE, 1939; CAPPELL, 1951). Neither theory has postulated any clear-cut mechanism by which these changes are brought about. An exception is the work of FELL (1949) who claims that an increased blood flow following sympathectomy increases the diameter of the central canal of the osteons. The majority of workers, however, continue to discount trophic control of the skeletal system, reporting no change following sympathectomy (BISGARD, 1931; KEY and MOORE, 1933; CORBIN and HENSEY, 1939). The present status has been concisely summarized by GILLESPIE (1954) who states that the diminished mechanical and dimensional parameters of bone following nerve injury are entirely due to "the loss of muscular power and tone, and hence disuse, and not the loss of any specific neurotrophic influence". The undoubted loss of osseous tissue in human cases of progressive facial hemiatrophy which do not show a concurrent loss of muscle activity continues to pose a problem. A tentative hypothesis, subject to experimental investigation, would state that normal bone growth depends on both the non-degeneration of muscle tissue, and on the continued normality of the total soft-tissue matrix within which the bone functionally exists.

With regard to our present data on the rat, we are unable to postulate a pathogenic mechanism for the observed fat loss. It does not help materially to note that such adipose-tissue atrophy is frequently observed clinically following nerve damage in the extremities. Possibly more detailed physiological methodologies would be fruitful. An indication of the difficulty of this sort of work is the report of EAYRS (1951) that bilateral, but not unilateral, sympathectomy of the week old rat caused delayed general somatic and hair growth. While we have experimentally implicated the sympathetic nervous system in the aetiology of progressive facial hemiatrophy, its pathogenesis remains enigmatic.

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FACIAL HEMIATROPHY FOLLOWING CERVICAL SYMPATHECTOMY IN THE RAT



FIG. 1. Ptosis of the upper *left* eyelid of a month-old rat immediately following successful cervical sympathectomy of that side.

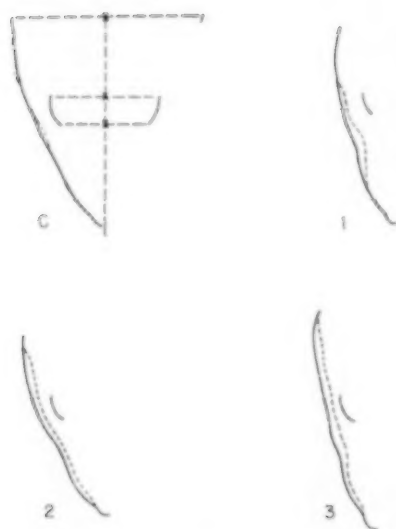


FIG. 2. Outline tracings of a typical control (C) and 3 animals with experimental facial hemiatrophy. In the control the method of determining the mid-sagittal line is illustrated. The solid line is the right side, the interrupted line is the left (operated) side. Note the normal asymmetry of the control and the uniform atrophy of the left side in the others.

FACIAL HEMIATROPHY FOLLOWING CERVICAL SYMPATHECTOMY IN THE RAT

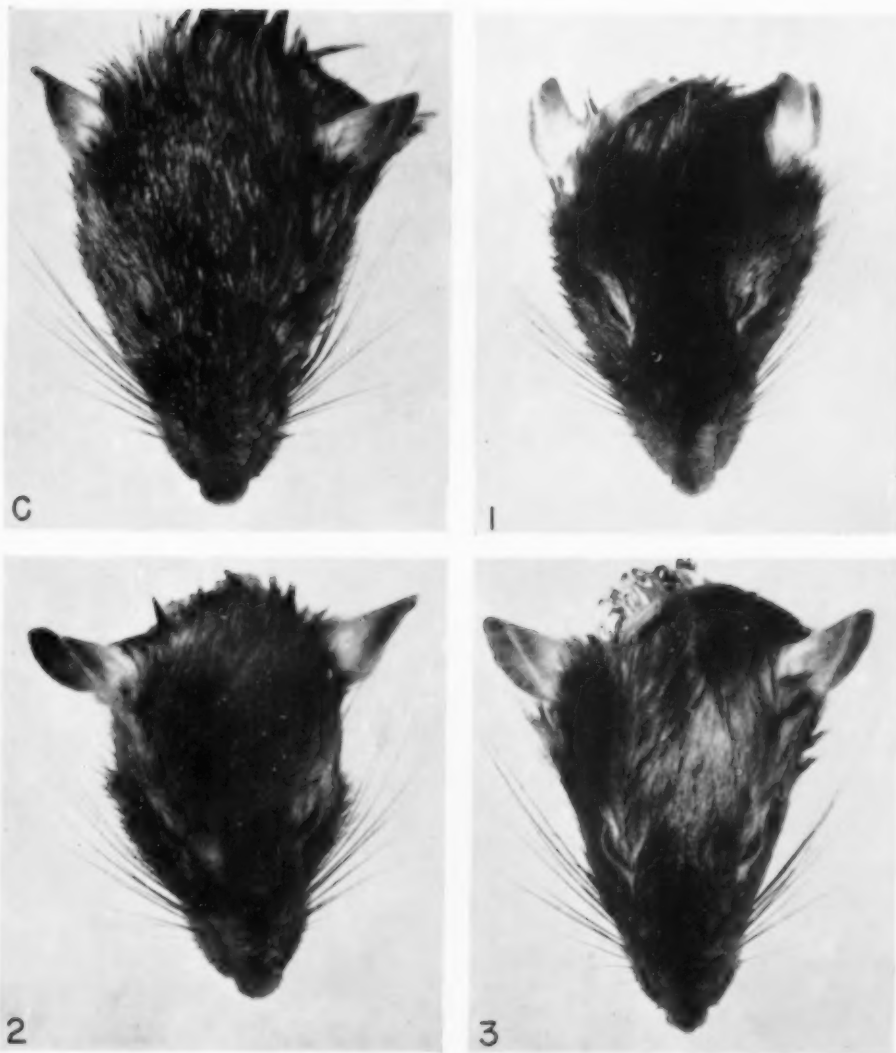


FIG. 3. The photographs from which the tracings in Fig. 2 were produced. Varying degrees of atrophy are shown on the 3 operated rats.

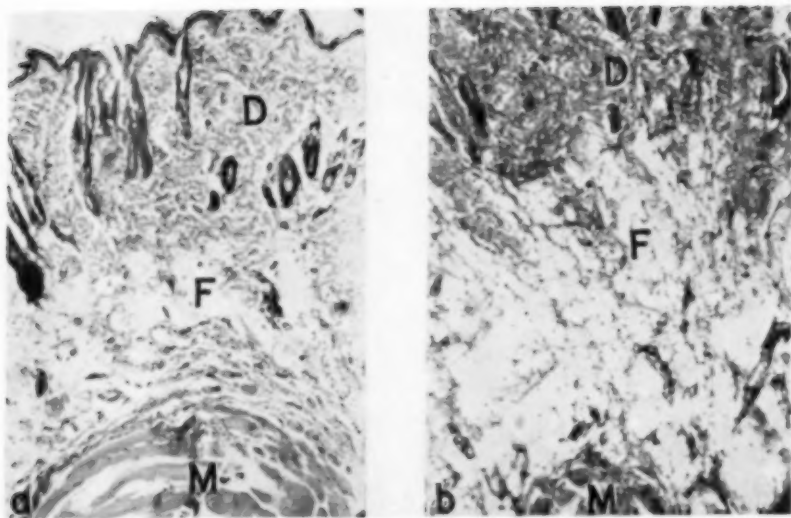


FIG. 4. Photomicrographs of identical areas of operated (a) and control (b) mystacial pads of a rat. The fat (F) is diminished on the operated side. (D=dermis; M=muscle.) Haematoxylin-eosin. $\times 83$.

REVIEW OF MODERN CONCEPTS ON CALCIFICATION

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Abstract—An important contribution of recent years towards calcification theories appears to be the epitactic concept, according to which bone salt crystallizes out from a supersaturated but otherwise stable tissue fluid on to a primary seed of the organic matrix. The close relationship between the fibre and mineral components shows that epitaxy is a distinct possibility in bony structures. It provides a better explanation of calcification than older theories, such as the precipitation theory of ROBISON, as it fits more readily the conditions prevailing in calcifying tissues. It accounts well for the role of adenosine triphosphate in crystal nucleation, and the importance of the organic matrix in mineralisation. Although the part played by the enzymes in bone salt deposition is small, epitaxy provides a reasonable explanation why enzymic inhibitors may have a considerable effect on bone salt turn-over.

THE outstanding contribution of recent years towards calcification mechanisms appears to be the epitactic concept of bone salt formation. This is a system in which the formation of bone salt is induced by a seeding agent. The significance of this theory is best seen in the light of its historical development (NEUMAN and NEUMAN, 1958). Regarding ROBISON's theory (1923) as the first modern view on calcification, the subsequent progress might be divided into the following phases: the implications of the alkaline phosphatase theory; importance of the organic matrix for calcification, and the concept of an epitactic stimulation of bone salt formation.

ROBISON visualized a precipitation of bone mineral from tissue fluids which became locally supersaturated with phosphate ions by the action of alkaline phosphatase on phosphate esters present in the calcifying area. This concept came under much criticism in subsequent years, because conditions in calcifying tissues are not favourable for a precipitation reaction. Another difficulty of the ROBISON theory was the lack of suitable substrates for alkaline phosphatase at the sites of calcification. ROBISON and ROSENHEIM (1934) met this objection by suggesting that organic phosphate compounds are formed from sugars occurring in areas of calcification, alkaline phosphatase being instrumental both in the formation and in the hydrolysis of these organic phosphate compounds. This assumption gained considerable support by the work of GUTMAN and his collaborators (1949, 1950) who showed a connexion between glycolysis, phosphate ester intermediates and calcification.

The next stage in the progress of calcification theories was the realization that the organic matrix was in some way involved in the mineralization process. Soon after ROBISON published his enzymic theory of calcification, it became evident that calcifying tissues possess qualities which distinguish them from those that do not calcify. Of the factors responsible for their distinctive behaviour, the phosphorylation

of the matrix and the role of the mucopolysaccharide moiety in the calcification process gained increasing importance.

Several workers found phosphate compounds to be present in excess of calcium in calcifying cartilage in the early stages of calcification (e.g. ROCHE, 1946), which suggested to them an interrelation between the phosphorus metabolism and ossification. Thus, GUTMAN and YÜ (1949) postulated that alkaline phosphatase transfers a phosphorylated intermediate of the glycolytic breakdown to an acceptor in the matrix, but they did not attempt to define the actual mechanism of mineral salt deposition. DALLEMAGNE (1948), however, has suggested an explanation for the calcification mechanism. He, like GUTMAN and YÜ, assumed a combination between the phosphate esters of glycolysis and the protein matrix of bone in the pre-calcification phase, and thought that, after the arrival of sufficient calcium ions at the site of calcification, phosphate ions were released from the protein-phosphate complex and precipitated as tricalcium phosphate. However, the validity of this idea has recently been questioned by WHITEHEAD and WEIDMANN (1959a), who showed that the phosphate turnover in this fraction was too low to account for such a rapid process as bone salt deposition.

Of the various phosphate esters contained in calcifying cartilage, adenosine triphosphate appears to be most closely linked with calcification. Not only were significant amounts of this substance found in cartilage (ALBAUM, HIRSHFELD and SOBEL, 1952), but POLONOWSKI and CARTIER (1951) also demonstrated rapid calcification in tissue slices of embryonic sheep cartilage in the presence of adenosine triphosphate, when in a similar experiment glucose phosphates gave only a slight effect. As a mechanism CARTIER and PICARD (1955) proposed a release of pyrophosphate from adenosine triphosphate which, by combining with the matrix, induces the formation of the first crystals of bone salt. Such an initiation of calcification by pyrophosphate nucleation is a typical instance of epitactic stimulation. According to this view, bone salt deposition occurs by a primary seed formation in the matrix on which hydroxyapatite crystallizes out from a supersaturated but otherwise stable tissue fluid.

Using a chromatographic technique, WHITEHEAD and WEIDMANN (1957) also demonstrated significant concentrations of adenosine triphosphate in the cartilage of kittens and puppies, and were able to show that the ^{32}P uptake of adenosine triphosphate is considerably higher than that of any other organic phosphate compound of the cartilage (WHITEHEAD and WEIDMANN, 1959a). The uptake was, in fact, of a similar magnitude to that found in the inorganic phosphate of bone salt, indicating an interconnexion between the two compounds. The importance of adenosine triphosphate for calcification received further support from experiments in which the effect of inhibitors on bone salt deposition was studied. On administering parathormone (WHITEHEAD and WEIDMANN, 1959b) or 2:4-dinitrophenol (WHITEHEAD and WEIDMANN, 1959c) to kittens, the uptake of ^{32}P was reduced in both the cartilage adenosine triphosphate and in the inorganic phosphate of bone salt. The effect was very quick in adenosine triphosphate and delayed, but gradually increasing, in inorganic phosphate. In the light of the modern seeding theory this might be

explained in the following way: being an enzymic process, adenosine triphosphate formation is immediately affected by interference with oxidative phosphorylation by parathormone or dinitrophenol, whereas the effect of these inhibitors on the turnover of bone salt is at first not apparent. At the time of inhibitor administration, there are still a sufficient number of crystal nuclei available for bone salt crystallization, but gradually the number of seeds is reduced due to the inhibition of adenosine triphosphate formation. This is then indicated by the decreasing rate of ^{32}P incorporation into inorganic phosphate, which depends on the new formation of bone salt crystals and the ionic exchange reaction between serum phosphate and the newly formed crystals.

The importance of epitaxy for bone salt deposition has been much stressed in recent publications (CARTIER and PICARD, 1955; NEUMAN and NEUMAN, 1958; IRVING, 1958). It has distinct advantages over the precipitation theory. For instance, no longer need it be assumed that bone salt deposition can occur only after alkaline phosphatase has produced a local supersaturation by phosphate ions in the tissue fluid, because crystallization, once induced by seed formation, follows spontaneously under the conditions prevailing in calcifying areas. It is envisaged that this process continues until there is no place for further crystal growth and mineralization is completed. Crystallization as a reaction needs less stimulation than precipitation (NEUMAN and NEUMAN, 1958).

The crucial problem then is whether the conditions are favourable for epitactic stimulation in a calcifying tissue. Has the collagen matrix itself epitactic qualities, or does crystal induction depend on the presence of a molecule associated with collagen? Numerous findings suggest a relation between the mineral and fibre components of bony tissue. ROBINSON and WATSON (1952) found a definite co-ordination between the fibre and mineral salt organizations of bone, and ROBINSON and CAMERON (1956) showed that bone salt is deposited in the banded regions of collagen. Hydroxyapatite crystals seem to be associated with the double bonds occurring at regular intervals along the fibre axis (FITTON-JACKSON, 1957). These facts apparently support the idea that the bone matrix has epitactic qualities, but they do not clarify the question whether the matrix needs activation in order to form a seed. It has been stated (STRATES, NEUMAN and LEVINSKAS, 1957) that collagen could induce epitactic stimulation but is not effective at physiological concentrations of calcium and phosphate ions in the tissue fluids. Only epitaxy is supposed to stimulate the fibres to bone formation.

Histochemical evidence clearly indicates that the organic matrix undergoes certain changes prior to calcification. Metachromasia and basophilia have been observed regularly in tissues about to calcify. These colour changes within the tissue were explained on the basis of molecular transformations of chondroitin sulphate (DZIEWIATKOWSKI *et al.*, 1957) which is also the substance most frequently connected with what has since been defined as the local factor. According to SOBEL (1952) the local factor is responsible for the calcifying ability of the tissue and for the orderly deposition of calcium salts into the matrix. He suggested that the local factor is a chondroitin sulphate-like substance, particularly well equipped for combinations

with calcium ions due to the presence of acidic and anionic groups in the molecule. SOBEL (1955) also demonstrated that at least part of the calcifying ability of cartilage is due to such a factor, because even after the sections were heated, completely destroying the enzyme systems of the phosphorolytic chain, calcium salts were still deposited. Considerable evidence accumulated in latter years from ^{35}S experiments showing that there is present in calcifying regions a compound capable of incorporating ^{35}S and possessing a reasonable activity (e.g. DZIEWIATKOWSKI, BENESCH and BENESCH, 1949; AMPRINO, 1955). More recently some doubts have been expressed as to whether chondroitin sulphate is the local factor, and the possibility was considered that other compounds might also be involved in the initiation of calcification. DZIEWIATKOWSKI *et al.* (1957) separated more than one sulphated polysaccharide from the epiphyseal plates of young rats, and IRVING (1958) assumed a mucopolysaccharide to act as a "primer" for calcification, but concluded from his histochemical tests that the substance was not chondroitin sulphate.

In spite of these doubts about the true nature of the local factor, the necessity for a stimulant to trigger off calcification was not questioned. As in the case of the phosphorylated matrix, the mucopolysaccharides might also require activation to initiate calcification. There is some evidence that this might indeed occur. In newly forming Haversian systems the innermost lamella, the one next to the Haversian canal, stained orthochromatically whereas the adjacent lamella was metachromatic and basophilic; the orthochromatic lamella was uncalcified, but the metachromatic lamella coincided with the beginning of calcification (WEATHERELL and WEIDMANN, 1959). It is of interest to note that in a similar system LACROIX (1956) demonstrated a primary uptake of ^{35}S in the non-calcified, orthochromatic lamella which was followed by a later entry of ^{35}S into the metachromatic lamella where calcification just commenced. This might suggest that a molecular transformation of a sulphated polysaccharide is responsible for ossification. It is quite possible that phosphorylation reactions play a part in this process, too. Thus SOBEL, BURGER and DEANE (1957) found that a treatment of chondroitin sulphate with adenosine triphosphate enhances calcification, leading him to assume an association between the phosphorylated matrix and the mucopolysaccharide moiety in the epitactic stimulation of bone salt deposition. This agrees with the hypothesis of ZAMBOTTI (1957), according to which a collagen-pyrophosphate-calcium-chondroitin sulphate complex is important for the seeding mechanism.

Oxidative enzyme systems and calcification

As seen from the foregoing discussion, much importance has been attached in modern seeding theories to the role of adenosine triphosphate, particularly where the mineralization of cartilage is concerned. In the past, the metabolism of cartilage was mainly considered as an anaerobic phenomenon. Due to the predominance of the alkaline phosphatase hypothesis which became connected with anaerobic carbohydrate metabolism, little notice was taken of a possible action of aerobic enzyme systems in calcifying cartilage. Cartilage was, in fact, regarded as an anaerobic tissue in which oxidative processes were thought to be almost completely absent.

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Gradually, however, some evidence was obtained of an oxidative metabolism. CASTELLANI and ZAMBOTTI (1954) demonstrated an increased concentration of succinic dehydrogenase in the layer of hypertrophic cells, and CARTIER and PICARD (1955) found a particularly high adenosine triphosphate content in this region, which happens to be the area where mineralization occurs. These authors (PICARD and CARTIER, 1956) thought, however, that adenosine triphosphate could only be produced anaerobically in view of the high glycolytic activity of the hypertrophying cells, and also because in their opinion the respiration of the cartilage was too low to synthesize such high concentrations of adenosine triphosphate as found in cartilage.

Anaerobic conditions are not particularly favourable for the synthesis of energy-rich phosphate esters which are produced in larger concentrations by aerobic reactions (LOOMIS and LIPMANN, 1948). Very recently WHITEHEAD and WEIDMANN (1959c) succeeded in demonstrating dehydrogenases and cytochrome oxidase in the hypertrophic zone of cartilage, and found that cartilage has a much bigger respiratory activity than was hitherto suspected. The respiration was stimulated by a variety of substrates, such as citrate, α -oxoglutarate, succinate, malate and pyruvate indicating that the Krebs cycle was operative in its entirety in cartilage. This shows that the tissue is well equipped with all the systems required for an active aerobic metabolism, and that the synthesis of adenosine triphosphate occurs, presumably by oxidative phosphorylation. Together with the results obtained from the experiments with parathormone and dinitrophenol, this leads to the conclusion that adenosine triphosphate plays an important part in bone salt formation by participating in the formation of a crystal seed. Although the nature of the seed is still unknown, a stimulation of crystallization by activating the collagen fibres or the mucopolysaccharide moiety seems likely. The activating mechanism might very well be a phosphorylation process, for which role adenosine triphosphate is especially suited due to its high energy content. The presence of the Krebs cycle, cytochrome oxidase and the cytochrome systems in calcifying cartilage adds further weight to this assumption.

These recent findings draw attention to some new aspects of the calcification process, but it would be premature to assume that there is now available an adequate explanation for the mechanism of calcification. Many questions are still insufficiently accounted for, such as the significance of glycolysis and its phosphorylated intermediates in the calcification reaction, the part played by alkaline phosphatase in calcifying tissues, the possibility of several seeding reactions and that more than one calcification mechanism operates in the various hard tissues.

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EFFECT OF PART-TIME EXPOSURE TO NATURAL WATER-BORNE FLUORIDES ON THE INCIDENCE OF DENTAL CARIES*

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Abstract—Evaluation was made of the effect on the incidence of dental caries in children of the consumption of fluorides occurring naturally in the drinking water either at home or at school. Full-time exposure to fluorides was not necessary to gain beneficial results. Ten per cent lower caries incidence than the control group was observed in all permanent teeth of a group of children consuming fluorides during school hours only. Eighteen per cent less caries was found in the premolar teeth of this group. Thirty-eight to seventy per cent less caries was found in another group of children consuming fluorides at home only. Here too the premolars demonstrated the greatest decrease (70 per cent) in DF surfaces.

THIS evaluation was undertaken to determine whether exposure of school children to natural fluorides (0.7–2.4 parts per million) in the drinking water for a portion of each day resulted in a significant decrease in the incidence of dental caries. This part-time exposure occurred at the school or in the home. The importance of such information can be appreciated when it is realized that approximately one-third of the population of the United States resides in areas which do not have a communal water supply. At the present time, there is no practical proven way of bringing the benefits of fluoridation to this group. The possibility of finding a solution to this problem by the utilization of methods of part-time fluoridation, such as fluoridation of the school water supply, prompted the initiation of this study.

Previous work with respect to the effect on dental caries of fluorides occurring naturally in the drinking water and also of fluorides added artificially to community drinking water supplies appeared conclusive. The Newburgh–Kingston Studies (AST *et al.*, 1956), the Brantford, Ontario (BROWN *et al.*, 1956; HUTTON, LINSBOTT and WILLIAMS, 1956), the Grand Rapids (ARNOLD *et al.*, 1956; HAYES, McCAULEY and ARNOLD, 1956), the Bartlett–Cameron (LEONE *et al.*, 1955), and the Evanston (HILL, BLAYNEY and WOLF, 1957; HILL, BLAYNEY and WOLF, 1956) studies all gave similar indications, i.e. that a reduction of 50–60 per cent in dental caries could be expected from continuous fluoridation of the community drinking water supply.

Work done in Pulaski, Tennessee (BRUCE and GUNTER, 1953) on a community water supply, intermittently containing amounts of natural fluorides varying between 0.3 and 1.3 parts per million (p.p.m.), showed a lower DMF rate there than was found in

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surrounding cities which had no fluorides in the drinking water. This indicated that continuous fluoridation at 1.0 p.p.m. might not be necessary to gain some of the benefits from fluoridation.

KLEIN (1948) noted an effect on the caries process from a part-time exposure of children to fluorides. He studied children who lived on farms and drank water (presumably low in fluoride content) from their own private wells. These children attended school in an area where the drinking water contained 1.2-2.2 p.p.m. of natural fluorides. The data presented for this portion of the study showed that such children experienced less caries than a group with a similar home water supply who attended schools in a fluoride-free area. However, no mention was made of radiographs to supplement the clinical examinations, nor were the water supplies at home analysed for fluoride content.

METHODS

In order to evaluate any decreases in dental caries that might result from part-time exposure to natural fluorides, the following controlled conditions were established:—

- (1) The areas investigated were those where no community drinking water supply existed.
- (2) The schools included in this study all had individual water supplies.
- (3) The drinking water of the experimental schools contained optimal amounts of naturally occurring fluorides which were constant over the past 5 years or more.
- (4) The water supplies of the control schools were free from fluorides for a similar period.

Schools and communities which satisfied these conditions were found in the rural areas of Rhode Island. Six schools were selected and 1140 children between the ages of 5 and 16 were examined. Three of the schools with a total of 515 students participating in the study had drinking water supplies between 1.0 and 1.3 p.p.m. of fluorides. One school in which 190 of the subjects were in attendance had 0.3 p.p.m. fluorides in the drinking water. Two schools in which 435 pupils were studied had no fluorides present in the drinking water. Table 1 shows the age distribution in these three categories.

Each child in the study was examined roentgenographically. A series of ten films was taken on each subject. These included three upper anterior periapical films, three lower anterior periapical films and two posterior bitewing films on each side.

All examinations were accomplished with mirror and explorer by the same examiner (J.A.Y.). The X-rays were viewed at the time of the clinical examination.

The examination data were recorded on 29 I.B.M. cards consisting of one "master card" and a series of 28 "tooth cards"—one tooth card for each of the teeth exclusive of the third molars.

The master card was used to record general personal information about the patient as well as the overall condition of the child's mouth with respect to hygiene, including tone of the tissues, and tooth stain.

TABLE 1. DISTRIBUTION OF SUBJECTS BY AGE AND FLUORIDE CONTENT OF SCHOOL DRINKING WATER

Fluoride content of school water	Age												All ages
	5	6	7	8	9	10	11	12	13	14	15	16	
1.0-1.3 p.p.m. "fluoride schools"	—	84	63	55	52	58	53	48	58	28	16	—	515
0.3 p.p.m.*	3	37	23	18	19	22	14	20	18	12	4	—	190
0.0 p.p.m. "no-fluoride schools"	7	64	53	62	54	43	45	33	43	22	5	4	435
	10	185	139	135	125	123	112	101	119	62	25	4	1140

* Subsequent statistical analyses revealed that the school with 0.3 p.p.m. fluorides in the drinking water exhibited a caries incidence equivalent to a 0.0 p.p.m. school. Thus, this school was classified as a "no-fluoride school".

Each tooth card was preprinted with the name of the tooth. The twenty-eight tooth cards were machine stacked in sequence running from the upper left to upper right and from lower left to right. The master cards and tooth cards were prepunched with specific codes representing school, grade, room, sex, age, and the child's own study code number.

At the time of examination, each individual tooth was listed on its respective tooth card, as to (a) type of dentition (primary or permanent), (b) condition of tooth (missing, sound, carious and/or filled) and (c) degree of stain. Each tooth surface involved by a caries process was listed as was every surface which showed a restoration. The depth of each carious lesion was also noted.

In addition, a sample of each subject's home drinking water was obtained. These were analysed for fluoride content by the Department of Sanitary Engineering of the State of Rhode Island according to the method of SCOTT-SANCHIS (1955).

To secure a complete record of the child's previous fluoride exposure, contact was made with each community in the United States, Canada and Hawaii which possessed a public water supply from which any child in the study had consumed drinking water for a year or longer during any period prior to present residence. In each case, information was obtained as to the fluoride content of these water supplies during the child's period of residence in the town or city.

This information was placed on I.B.M. cards along with the additional data obtained from a detailed history form secured on each child. These cards were processed by the Division of Statistical and Research Studies at Boston University, Boston, Massachusetts, and subjected to statistical analysis.

RESULTS

Assays of fluorides in the home water supplies (wells) showed that 313 children were consuming varying amounts of fluorides at home. Of these, 104 children had home water supplies containing fluorides in excess of 0.7 p.p.m. This necessitated dividing the population for study purposes into groups determined by the fluoride exposure of the child and resulted in the groupings shown in Table 2.

TABLE 2. GROUPS DETERMINED BY FLUORIDE EXPOSURE

Group	F ⁺ content of water		Description	No. in group
	Home	School		
1	—	—	No significant fluoride exposure	372
2	—	+	Exposure to fluorides at school*	129
3	+	—	Exposure to fluorides at home†	36
4	+	+	Exposure to fluorides at home and school	68
5			Other fluoride groups	
a	—	Trace	(a) No home exposure and less than 4 years at school	205
b	Trace	—	(b) No school exposure and exposure to less than 0.7 p.p.m. at home	209
c	Trace	Trace	(c) Exposure to less than 0.7 p.p.m. at home and less than 4 years exposure at school	121

* Fluoride exposure at school was defined as exposure to 1.0-1.3 p.p.m. for a period longer than 4 years. This time requirement was set in order to allow the canine, premolar and second molar teeth sufficient exposure to fluorides in their pre-eruptive stages to gain the benefits of fluoridation.

† Fluoride exposure at home signified exposure to amounts of fluorides in excess of 0.7 p.p.m. for a period greater than the first 6 years of life. The breakoff figure of 0.7 p.p.m. was used since most public health authorities feel that fluorides should ideally be 0.7 p.p.m. or greater to demonstrate a significant reduction in the incidence of dental caries.

These groups could be further subdivided according to age. Table 3 shows the breakdown by age groups of each category which had received significant fluoride exposure. Also shown are the mean DF surfaces for the anterior, premolar and molar teeth, and the total mean DF surfaces for each age group.

For comparative purposes, group 1 with no significant fluoride exposure served as the control reference. Group 4, which represented virtual full-time fluoride exposure, showed an average caries reduction for anterior, premolar and molar teeth of 43 per cent. It was felt that further analysis of this group would add little to previous findings on full-time fluoridation. Consequently, no other analysis will be presented on this portion of the data.

Group 5, which had minimal fluoride exposure, i.e. exposure to what is considered less than optimal concentration of fluoride ions, was eliminated from the present analysis. A subsequent report on this phase of fluoridation is planned. Groups 2

TABLE 3. DF SURFACES OF GROUPS EXPOSED TO SIGNIFICANT LEVELS OF FLUORIDES

Group		No. in sample	Mean age (months)	Total DF surfaces	Mean DF surfaces		
					Anteriors	Premolars	Molars
1	No significant fluoride exposure						
	Ages 5-9*	201	93.8	5.91	0.69	0.21	5.01
	10-13	143	144.2	15.59	2.80	2.57	10.21
	14 and over	28	175.4	26.21	5.54	6.29	14.39
		372					
2	Fluoride exposure at school only (more than 4 years)						
	Ages 5-9†	—	—	—	—	—	—
	10-13	105	145.2	14.08	2.48	2.11	9.35
	14 and over	24	176.3	21.25	5.21	5.04	11.00
		129					
3	Fluoride exposure at home only (at least 0.7 p.p.m.)						
	Ages 5-9	19	89.5	3.21	‡	‡	3.75
	10-13	14	136.4	10.07	1.57	0.86	7.64
	14 and over	3	125.3	19.67	4.67	3.67	11.33
		36					
4	Home and school fluoride exposure						
	Ages 5-9	44	93.8	3.43	0.25	0.14	3.05
	10-13	19	145.9	10.63	2.37	0.95	7.32
	14 and over	5	179.2	23.81	8.60	7.00	8.20
		68					

* Throughout this study the 5-9 age group included children from 5 years to 9 years and 11 months. The 10-13 year group included children from 10 years to 13 years and 11 months.

† Children in this age group would have received less than 4 full years' exposure to fluorides at school.

‡ Sample too small for analysis.

and 3 represented a part-time exposure to fluorides. These groups were studied in detail and comparisons were made with the control group.

The results of these detailed comparisons showed a consistent quantitative relation, i.e. in every instance a definitely lower caries rate was found among children consuming optimal concentrations of fluorides in only a part of each day's total water consumption.

For the first comparisons two composite groups of children were formed. One group was composed of those children with insignificant fluoride exposure (control).

This group included those children with no home exposure and less than 4 years' exposure to fluorides at school. A second group included those children with high fluoride exposure without regard for its source (i.e. home or school). Comparisons were made of the incidence of caries in those two groups. These comparisons were made separately for all permanent teeth, anterior teeth, premolar and molar teeth. Table 4 shows the results of these comparisons based on DF surfaces.

TABLE 4. COMPARISON OF CARIES INCIDENCE BETWEEN CONTROL GROUP (NO HOME FLUORIDE EXPOSURE AND LESS THAN 4 YEARS' SCHOOL EXPOSURE) AND COMPOSITE GROUPS (SIGNIFICANT FLUORIDE EXPOSURE WITHOUT REGARD TO SOURCE OF FLUORIDES—SCHOOL OR HOME)

(a)								
Control group					Fluoride exposed group			
Age group	No. in sample	Mean age (months)	Mean DF surfaces with standard deviations		No. in sample	Mean age (months)	Mean DF surfaces with standard deviations	
			All permanent teeth	Anterior teeth			All permanent teeth	Anterior teeth
5-16*	577	114.7	9.91 (9.97)	1.60 (3.28)	104	114.7	7.05 (7.88)	1.32 (3.25)
5-13†	542	110.7	8.83 (8.61)	1.35 (2.94)	96	109.5	5.78 (6.00)	0.83 (2.43)
5-9	354	93.5	5.42 (5.37)	0.63 (2.19)	63	92.5	3.37 (3.28)	0.21 (0.93)
10-13	188	143.2	15.25 (9.80)	2.69 (3.61)	33	141.9	10.39 (9.21)	2.03 (3.66)
(b)								
Control group					Fluoride exposed group			
Age group	No. in sample	Mean age (months)	Mean DF surfaces with standard deviations		No. in sample	Mean age (months)	Mean DF surfaces with standard deviations	
			Premolar teeth	Molar teeth			Premolar teeth	Molar teeth
5-16	577	114.7	1.29 (3.06)	7.04 (5.27)	104	114.7	0.79 (2.40)	4.94 (3.98)
5-13	542	110.7	0.96 (2.48)	6.54 (4.85)	96	109.5	0.38 (1.17)	4.59 (3.75)
5-9	354	93.5	—	4.64 (3.56)	63	92.5	—	3.06 (2.69)
10-13	188	143.2	2.45 (3.66)	10.11 (4.95)	33	141.9	0.91 (1.71)	7.45 (3.81)

* Including children from 5 years to 16 years and 11 months.

† Including children from 5 years to 13 years and 11 months.

For all permanent teeth, such comparisons revealed a 29 per cent lower caries rate in children of all age groups with high fluoride exposure. When the permanent teeth of the 5-9 year old children were evaluated in this manner, a 38 per cent lower caries rate was found in the group consuming significant amounts of fluorides. Thirty-two per cent less caries of all permanent teeth was seen in the 10-13 years age group in a like comparison.

The anterior, premolar and molar teeth followed a similar pattern. The 5-13 year old children who consumed significant amounts of fluorides showed a 39 per cent lower caries rate for anterior teeth than the control group. This group also exhibited

a 60 per cent lower caries rate than the control group for premolar and a 30 per cent lower rate for molar teeth.

Significantly lower caries rates were also found for the anterior, premolar and molar teeth in the 5-9 and 10-13 year old subjects in this composite group.

The next comparisons were made between a control group which had virtually no fluoride exposure (Group 1) and the groups receiving substantial exposure to fluorides either only at home or at school. Such an analysis revealed even greater differences in the incidence of caries than those seen in the composite groups. Thus, children 5-13 years of age exposed to optimal fluoride concentrations at home but with no significant school fluoride exposure had 38 per cent less caries in the permanent teeth than the control group. The 5-9 year old children in this category who had fluoride exposure showed a 46 per cent lower caries incidence than the control group. The difference was 35 per cent for the 10-13 year old children who were exposed to significant amounts of fluorides in their home drinking water.

TABLE 5. COMPARISON OF CARIES INCIDENCE BETWEEN CONTROL GROUP (1) AND GROUPS RECEIVING PART-TIME EXPOSURE TO CONCENTRATIONS OF FLUORIDES IN EXCESS OF 0.7 p.p.m. EITHER AT SCHOOL OR AT HOME (GROUPS 2 AND 3)

(a)

Control group (1)					Fluoride exposure at home only (3)			
Age group	No. in sample	Mean age (months)	Mean DF surfaces with standard deviations		No. in sample	Mean age (months)	Mean DF surfaces with standard deviations	
			All permanent teeth	Anterior teeth			All permanent teeth	Anterior teeth
5-13	344	114.8	9.93 (9.01)	1.57 (3.14)	33	109.5	6.12 (5.69)	0.73 (2.29)
5-9	201	93.8	5.91 (5.57)	0.69 (2.34)	19	89.5	3.21 (2.63)	—
10-13	143	144.2	15.59 (9.86)	2.80 (3.66)	14	136.4	10.07 (6.31)	1.57 (3.29)
Control group (1)					Fluoride exposure at school only (2)			
10-13	143	144.2	15.59 (9.86)	2.80 (3.66)	105	145.2	14.08 (8.47)	2.40 (3.51)

(b)

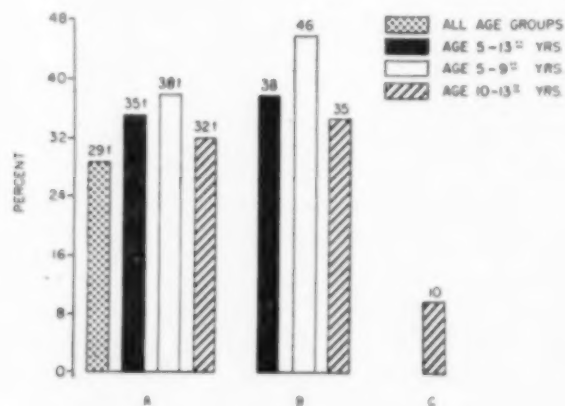
Control group (1)					Fluoride exposure at home only (3)			
Age group	No. in sample	Mean age (months)	Mean DF surfaces with standard deviations		No. in sample	Mean age (months)	Mean DF surfaces with standard deviations	
			Premolar teeth	Molar teeth			Premolar teeth	Molar teeth
5-13	344	114.8	1.19 (2.80)	7.17 (4.82)	33	109.5	0.36 (0.98)	5.03 (3.90)
5-9	201	93.8	—	5.01 (3.57)	19	89.5	—	3.11 (2.63)
10-13	143	144.2	2.57 (3.83)	10.21 (4.71)	14	136.4	0.86 (1.36)	7.64 (3.81)
Control group (1)					Fluoride exposure at school only (2)			
10-13	143	144.2	2.57 (3.83)	10.21 (4.71)	105	145.2	2.11 (2.78)	9.35 (4.66)

As in the composite group analyses, children exposed to fluorides only at home showed a significantly lower caries incidence than the control group for anterior teeth, premolars and molars. A 53 per cent lower caries rate was found for the anterior teeth of these children in the 5-13 year age group.

The premolars showed the most dramatic effect. The 10-13 year old children were studied since most of the premolar teeth would be found in children of this age group. Analysis revealed 67 per cent less caries in premolar teeth in the children drinking fluoridated water only at home. A 25 per cent lower rate than the control group was found for molar teeth of 10-13 year old children in a similar comparison.

The analysis of the teeth of children who received their part-time exposure to fluorides only at school was of special interest. It was found that here too the benefits of fluoridation are evident. Compared to the control group, 10 per cent less caries was found in all permanent teeth of the children receiving fluoride exposure at school only.

The premolars continued to show the most notable evidence of fluoridation exhibiting 18 per cent less caries in the 10-13 year age group with just school fluoride exposure. The molars of these children with school fluoride exposure showed 9 per cent less caries than the group with no fluoride exposure. Table 5 shows the details of the comparisons between the control group and the group receiving fluoride exposure at home or at school. The above information is pictured graphically in Figs. 1, 2, 3 and 4.



(1) THE FIGURES 9⁰⁰ AND 13⁰⁰ REPRESENT 9 YEARS AND 11 MONTHS AND 13 YEARS AND 11 MONTHS.

0 1-TEST SIGNIFICANT TO 1% LEVEL OR BEYOND

FIG. 1. Percentage reduction of DF surfaces on all permanent teeth in groups with significant fluoride exposure compared with control groups in corresponding age categories. (A) Composite group of all children receiving significant fluoride exposure without regard for the source (home or school). (B) Group receiving exposure to fluorides at home only. (C) Group receiving exposure to fluorides at school only.

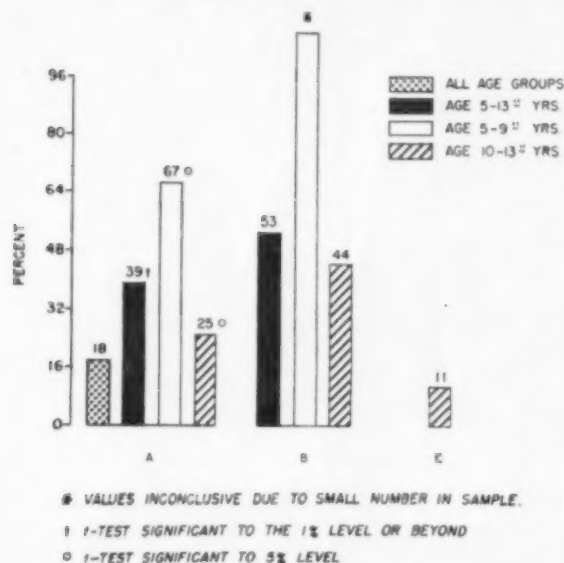


FIG. 2. Percentage reduction of DF surfaces on anterior teeth in groups with significant fluoride exposure compared with control groups in corresponding age categories. (A) Composite group of all children receiving significant fluoride exposure without regard for the source (home or school). (B) Group receiving exposure to fluorides at home only. (C) Group receiving exposure to fluorides at school only.

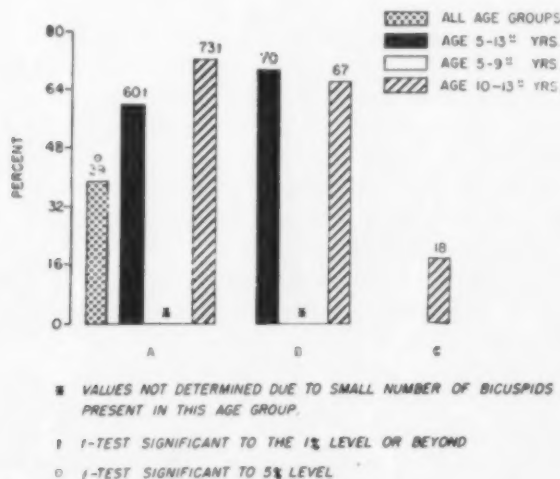
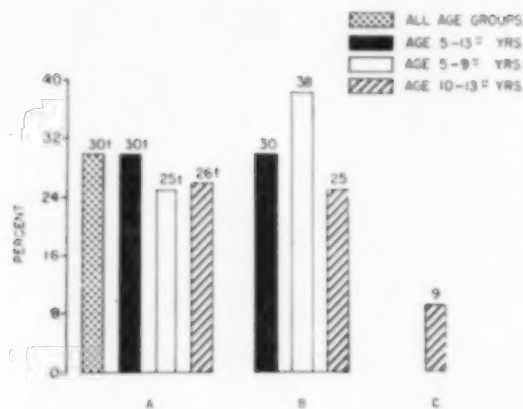


FIG. 3. Percentage reduction of DF surfaces on premolar teeth in groups with significant fluoride exposure compared with control groups in corresponding age categories. (A) Composite group of all children receiving significant fluoride exposure without regard for the source (home or school). (B) Group receiving exposure to fluorides at home only. (C) Group receiving exposure to fluorides at school only.



† - t-TEST SIGNIFICANT TO THE 1% LEVEL OR BEYOND

FIG. 4. Percentage reduction of DF surfaces on molar teeth in groups with significant fluoride exposure compared to control group in corresponding age categories. (A) Composite group of all children receiving significant fluoride exposure without regard for the source (home or school). (B) Group receiving exposure to fluorides at home only. (C) Group receiving exposure to fluorides at school only.

DISCUSSION

It is apparent from the results obtained that a demonstrable reduction in the incidence of dental caries occurs with "full-time" fluoridation of the drinking water and that definite benefits are obtained with less than full-time exposure of children to optimal amounts of fluoride salts. The effect of a part-time exposure to optimal amounts of fluorides appears to vary proportionately with the amount of fluorides ingested. Thus, children consuming fluorides in the home drinking water could be expected to ingest a greater amount of fluorides than children drinking only fluoridated school water. This is borne out by the greater reduction of caries in the former group (38 per cent for all teeth and 70 per cent for premolars) as compared to the group of children consuming fluoridated water only at school (10 per cent less caries for all teeth and 18 per cent less for premolars).

However, the fact that a definite reduction in caries can occur when fluorides are consumed only during school hours shows that only a relatively low volume of water need be ingested to obtain a definite effect on the caries process provided the fluorides are present in the drinking water in optimal concentrations, i.e. amounts greater than 0.7 p.p.m.

The small difference (9 per cent) in the caries rates of molar teeth between children who drank fluoridated water only at school and the control group may be due to inclusion of both first and second molars in the statistical analysis. In most children the first molar would have erupted before any benefit could be derived from the consumption of fluoridated school water. Further detailed study of the canine and second molar teeth is planned.

The following conclusions may be drawn from this study concerning the effect on the incidence of dental caries of the part-time exposure of school children to natural water-borne fluorides:

- (1) The findings of caries reduction from the consumption of fluorides in the drinking water are upheld.
- (2) Consumption of water containing optimal amounts of fluorides for only part of the day will result in a decrease in the incidence of caries.
- (3) The reduction in caries appeared to vary proportionately with the amount of fluorides ingested, i.e. the less the exposure to 1.0 p.p.m. of fluoride containing drinking water, the less the apparent caries reduction.
- (4) Even the small amounts of fluorides consumed at school when no fluorides are ingested at home resulted in a definitely lower caries incidence—10 per cent for all teeth and 18 per cent for premolar teeth in this study.
- (5) The greatest effect of part-time fluoride exposure appeared to be in the premolar teeth—18 per cent less caries than the control group from school exposure alone, and 70 per cent less caries from home exposure alone.

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PRESERVATION AND STORAGE OF ORAL TISSUES—I

PRESERVATION OF TOOTH GERMS BY FREEZING, EVALUATED BY CELLULAR OUTGROWTH IN TISSUE CULTURES

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Abstract—The effectiveness of glycerol and ethylene glycol in preserving the viability of tooth germs subjected to various freezing temperatures was investigated by explanting the tissues, after thawing, to tissue cultures, and observing the extent of cellular outgrowth and the degree of internal differentiation. Tooth germs were pretreated with various concentrations of the preservative agents, frozen indirectly in dry ice-alcohol solutions (-78°C), isopentane cooled in liquid nitrogen (about -120°C), or liquid nitrogen (-198°C), and cultured in Carrel flasks. Glycerol in 30% solution appeared to be the most effective pretreating agent, and the slowest rate of freezing (dry ice-alcohol at -78°C) was the least damaging of the freezing rates employed.

INTRODUCTION

THE preservation and storage of tissues for future use in replacement techniques have become important adjuncts to therapeutic practice. Although storage is not necessarily a prerequisite for transplantation procedures, it affords obvious advantages in many types of grafting operations. The ideal preserved graft has been described as one which, after storage and implantation, immediately and permanently fulfils all the normal physiologic functions of the replaced tissue (PATE, 1954). Despite the implication that viability is a necessary requisite of ideal grafts, in actual practice viability often may be of secondary or even of no importance. In these cases the function of the so-called "homostatic" graft is essentially mechanical, and the graft includes such tissues as bone and cartilage, while the "homovital" graft is dependent on the viability of the tissue and its ability to perform its essential functions.

The necessity for viability in tooth implantation has been questioned by some (KUPFER and KUPFER, 1953) and supported by others (TAM, 1956). Reports of satisfactory results with essentially non-viable transplants have emphasized reattachment and mechanical function (KUPFER and KUPFER, 1953; PAFFORD, 1956). However, the results usually seen when partially formed or developing teeth have been autogenously transplanted (CLARK, TAM and MITCHELL, 1954; TAM, 1956) appear to favour the use of viable over nonviable tooth-elements in implantation techniques, where this is possible.

Following the methods initiated by POLGE, SMITH and PARKES in 1949, the successful preservation of viable tissues by freezing after treatment with glycerol has been reported for a number of tissues and organs (WOLSTENHOLME and CAMERON, 1954). In general, however, attempts at the preservation of vital teeth have proved relatively unsatisfactory so far, probably owing as much to the infrequency of investigation into the problem as to other reasons. However, our preliminary trials with the Parkes techniques applied to foetal oral tissues (GERSTNER and BUTCHER, 1958a) indicated the value of a more comprehensive examination of these methods. This paper reports the results of the first in a series of investigations dealing with the effects of freezing agents and freezing rates on viable oral tissues.

METHODS

Molar and incisor tooth germs were removed under sterile conditions from 15- to 20-day rat foetuses (i.e. before the formation of hard tissues), with a dissecting microscope following in general the Glasstone technique as modified by LEFKOWITZ and MARDFIN (1954). The tooth germs, consisting of either papilla and enamel organ, or pulp and enamel organ, were maintained in physiological saline solutions to prevent dehydration pending freezing and culturing.

Freezing and thawing

Three different freezing agents were used: liquid nitrogen (-198°C), isopentane cooled in liquid nitrogen (-120 to -140°C), and solid carbon dioxide (dry ice) dissolved in ethyl alcohol (-78°C). Since in these experiments the temperatures essentially determined the rates of freezing, the freezing methods are arbitrarily referred to as rapid, intermediate and slow methods, respectively. The tooth germs to be frozen were put on sterile wood-splints and placed in test tubes in such a way that the tissues did not come in contact with the glass. The test-tubes were then sealed and immersed in the freezing solutions for the following minimum times: liquid nitrogen—3 min; isopentane cooled in liquid nitrogen—3 min; and dry ice in ethyl alcohol—15 min. Immediately upon their removal from the freezing agents the tooth germs were rapidly thawed in Tyrode solution and retained in the physiological saline until ready for culturing.

Glycerol pretreatment

Tooth germs were immersed for 5, 10 and 15 min in concentrations of 30% and 60% glycerol by volume in Tyrode solution. Immediately following this treatment the tooth germs were frozen and thawed as described above. After thawing they were washed in four changes (each 15 min in duration) of physiological saline to remove the glycerol before culturing.

Ethylene glycol pretreatment

Tooth germs were similarly pretreated with concentrations of 15%, 20% and 30% ethylene glycol in Tyrode solution. Freezing and thawing methods were the same as for glycerol and the tissues were washed free of the glycol solution prior to culturing.

Culturing

All tooth germs were cultured in 3.5 cm Carrel flasks using standard tissue-culture methods (CAMERON, 1950). Most tissues were grown in a coagulated medium of chick plasma, rat- or chick-embryo extract and Earle's physiological saline solution, with penicillin and dihydrostreptomycin added to ensure sterility. The tooth germs were fed every second day with a nutrient fluid of Earle's solution, embryo extract and horse serum, and were maintained at a temperature of approximately 36°C and a pH of about 7.2 for as long as 3 weeks. Observations were made daily.

Since a rapid method for determining the viability of the tooth germs after freezing was considered desirable, environmental conditions in the cultures were adjusted to promote epithelial outgrowth from the enamel organ (GERSTNER and BUTCHER, 1958b). Results were evaluated chiefly by the extent and condition of this cellular outgrowth from the cultured tissues. The extent of outgrowth from untreated control tooth germs was designated as grade 4. Grade 0 represents no growth whatsoever. Grades 1, 2 and 3 stand for outgrowth approximately 25, 50 and 75 per cent respectively as extensive as that of the untreated control samples of tooth germs incubated for the same length of time.

Differentiation within the explants was examined histologically and the degree of development was compared with previously determined results (GERSTNER and BUTCHER, 1958b).

OBSERVATIONS

Non-pretreated, non-frozen controls

These controls, grade 4, represented the standard, e.g. extent of outgrowth, condition of the cells, viability of the tissue, against which the treated tissues were evaluated. In general, cultures of the non-pretreated, non-frozen tooth germs showed an extensive epithelial outgrowth which appeared about 24 hr after culturing (Fig. 1), from essentially all parts of the tissue (Fig. 2). The cells were healthy and normal and maintained this state throughout the period of culturing. The tooth germs remained intact and their rate of growth generally corresponded to that previously shown by GERSTNER and BUTCHER (1958b).

Pretreated, non-frozen tooth germs

Tooth germs treated with 30% glycerol in Tyrode solution for 10–15 min differed little from the untreated controls. In most cases the cellular outgrowth, appearing about 1.5 days after culturing (Fig. 1), was almost equal to that from the non-pretreated, non-frozen controls, and the average rating for the group was grade 3.7. The epithelial cells were normal, healthy and adhered to each other in the typical form of epithelial sheets (Fig. 3). Treatment with a concentration of 60% glycerol in Tyrode was followed by less satisfactory results. The extent of the outgrowth was decreased and the epithelial cells, while differing little cytologically from the controls, partially lost their characteristic adherent quality during migratory movements.

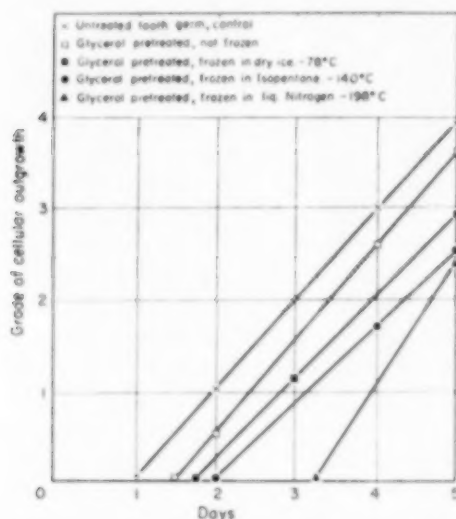


FIG. 1. The inhibiting effects of different freezing agents and rates of freezing on both the time of initial appearance and the extent of the cellular outgrowth. Extent of outgrowth is graded at 1-4; control=grade 4.

In cultures of tooth germs with 20% concentrations of ethylene glycol in Tyrode for 10-20 min the average extent of cellular outgrowth was about grade 3-5. The cells were healthy and adherent although somewhat more granular than the controls, and migrated in regular sheets (Fig. 4). Tooth germs treated with 15% and 30% concentrations of ethylene glycol did not show as good results. After a concentration of 30%, cellular outgrowth was significantly decreased and frequently failed to maintain typical epithelial sheet formation. Individual cells were more granular and lacked the uniformity associated with healthy-tissue culture growth.

Frozen, non-pretreated tooth germs

Tooth germs frozen without protective pretreatment did not usually survive when thawed and transferred to the culture flasks (Fig. 5). Pulps were often distorted; some were either withdrawn completely into and away from the wall of the enamel organ, or were torn away and extruded from the rest of the tooth germ. It was observed with all tooth germs of this group that the lower the temperature of the freezing agent, the greater was the damage. In several instances following the freezing of non-pretreated tooth germs in a dry ice-ethyl alcohol mixture (-78°C , the highest freezing temperature used), a few cells appeared in the cultures (Fig. 6).

Glycerol-pretreated and frozen tooth germs

Tooth germs thawed and cultured after pretreatment with 30% glycerol in Tyrode solution and freezing in a dry ice-ethyl alcohol mixture (-78°C) for 15-30 min, showed an average cellular outgrowth of approximately grade 3-0. The epithelial

cells, which first appeared at an average of 1.8 days after culturing (Fig. 1) were in healthy condition; they migrated in typical sheets, were not excessively granular and were relatively uniform in size (Fig. 7). The tooth germs and the general pattern of the outgrowth tended to resemble the non-frozen 30% glycerol-treated controls except that cellular outgrowth proceeded at a slower rate.

Cultures of tooth germs frozen in isopentane (-120 to -140°C) after pretreatment with 30% glycerol in Tyrode solution for 15 min had an average cellular outgrowth of about 2.6. The cells, first seen after 2 days of culturing (Fig. 1) were more granular, less uniform, and migrated in more irregular sheets than cells from similarly treated tissues frozen at -78°C (Fig. 8). Tooth germs frozen in liquid nitrogen (-198°C) after pretreatment with 30% solutions of glycerol in Tyrode for 5–15 min, thawed and cultured, showed an average outgrowth of about grade 2.4. The cellular outgrowths, appearing after 3.3 days of culturing (Fig. 1), were not as extensive or as healthy as the glycerol-pretreated tissues frozen at -78°C and -120 to -140°C . The cells were granular: the epithelial sheets were irregular and did not migrate from all parts of the tooth germ (Fig. 9). Those tooth germs, pretreated with 60% concentrations of glycerol in Tyrode solution and frozen at -198°C , showed even less favourable results. Outgrowth was much less extensive and consistently irregular; the cells were of various sizes and shapes and more granular than the 30% glycerol-treated group frozen at -198°C .

Ethylene glycol pretreated, frozen tooth germs

Cultures of tooth germs pretreated with a 20% solution of ethylene glycol in Tyrode for 15–20 min prior to freezing in dry ice (-78°C) for 20–25 min, showed an average cellular outgrowth graded at 2.5. Initially the outgrowth formed adherent

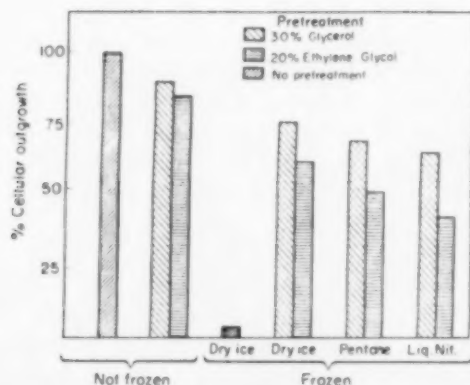


FIG. 10. The effectiveness of two protective pretreatments (30% glycerol in Tyrode solution, 20% ethylene glycol in Tyrode solution), in preventing damage to tooth germs subjected to three different rates of freezing (dry ice in ethyl alcohol at -78°C , isopentane at -120 to -140°C , and liquid nitrogen at -198°C); evaluated by the extent of cellular outgrowth in tissue cultures. Cellular outgrowth of controls = 100%.

epithelial sheets, and individual cells were only a little more granular than the non-frozen, ethylene-glycol-treated controls. However, in 2-3 days scattered migratory activity was seen, tending to disrupt the uniform epithelial sheet formations. The cells became irregular and more granular. These effects were more pronounced when the tissues were pretreated with 30% concentrations of ethylene glycol before freezing at the same temperature.

Cultures of tooth germs pretreated with 20% concentrations of ethylene glycol and frozen in isopentane at -120 to -140°C and in liquid nitrogen at -198°C showed progressively poorer results in proportion to the lower temperatures. Outgrowths from the pretreated isopentane frozen tissues were graded at 2.0 and generally failed to maintain adherent epithelial sheet formation. The cells were heavily granular, of irregular sizes and shapes, and migrated singly or in groups. The cultures of the liquid nitrogen-frozen tooth germs, pretreated with ethylene glycol, produced the poorest results of the experimentally treated tissues. The average cellular outgrowth was graded approximately 1.7. Outgrowths were irregular; the epithelial sheets were inconstant and tended to disintegrate, soon after appearing, into scattered groups of migrating cells. These cells were generally large, granular and non-adherent.

Differentiation

Internal differentiation tended to parallel cellular outgrowth. Untreated controls compared favourably with growth levels previously established (GERSTNER and BUTCHER, 1958b). The grades representing the extent of cellular outgrowth could in general be applied to the degree of internal development in the treated tooth germs. However, differentiation proceeded at a considerably slower rate than cellular outgrowth, reaching an approximately similar grade 1-2 weeks later than the latter.

The effectiveness of the two more favourable preservation methods in protecting tooth germs from the damaging effects of low temperatures is compared in Fig. 10. A combination of 30% glycerol pretreatment and slow freezing with dry ice in alcohol at -78°C afforded the greatest protection to the tooth germs as measured by the percentage of cellular outgrowth, where the average outgrowth from non-pretreated, non-frozen controls was rated at 100 per cent. Glycerol appeared to be the more effective pretreating agent, and the slowest rate of freezing (dry ice-ethyl alcohol mixture at -78°C) the least damaging of the freezing rates employed.

DISCUSSION

The considerable research currently being conducted in the transplantation of oral tissues by FLEMING (1955, 1956), PAFFORD (1956), WAITE (1956) and others emphasizes the necessity for more detailed investigations of methods for preserving and storing dental tissues for future availability. The effectiveness of glycerol and "slow" freezing rates as a method of protecting viable tooth germs from the damaging effects of low temperatures is in accord with similar findings for other tissues (SMITH, 1954; TAYLOR and GERSTNER, 1955) and warrants its use as a base for further exploration for dental applications. Although other tissues frozen without pretreatment have shown little viability when grown *in vitro* (TAYLOR and GERSTNER, 1955),

similarly treated tissues implanted back into the animal as autografts have been reported to survive in some cases (BILLINGHAM and MEDAWAR, 1952). There is little doubt that the autologous *in vivo* environment is more conducive to growth than an *in vitro* or tissue-culture environment. The latter, however, is a convenient medium for comparison, and since normal untreated tissues ordinarily grow well in cultures, the effect of freezing without pretreatment must be to damage cells enough to inhibit their growth in this medium. Pretreatment with glycerol significantly protects the cells from these effects. Ethylene glycol, an adequate protective agent only under limited conditions, appears to be more toxic to the cells than glycerol, in the concentrations used here.

PAFFORD (1956) and WAITE (1956), using preserved adult teeth for transplantation experiments, observed that the use of liquid nitrogen (-198°C) as a freezing agent resulted in the cracking and splitting of the enamel. PAFFORD also reported that his best results followed freezing and storage of adult teeth at -30°C , and believed that he had transplanted viable teeth. Although this appears to be additional confirmation of the advantages of slow freezing, it is not certain that viability was maintained for any considerable period in his tissues at this temperature. LOVELOCK (1953a,b), TAYLOR and GERSTNER (1955) and others have demonstrated that the range from approximately -5°C to -40°C is a critical region of temperature in which non-pretreated cells are usually irreversibly damaged if they remain longer than a few seconds. Pretreated tissues can be protected against such damage in this range for many hours, but probably not for the extended periods of time that storage would involve (SMITH, 1954).

A positive correlation appears to exist between the time of appearance of the first cells in the cultures and either the freezing temperature or the freezing rate. The lower the freezing temperature, the greater was the delay in the initial appearance of cellular outgrowth in the pretreated cultures (Fig. 1), indicating either an inhibiting effect on the return to normality of the cells, or the death of a greater number of cells. Probably both factors were operating. This is emphasized by the results in the cultures of the frozen but non-pretreated tooth germs. After a considerable delay, a few living cells were observed in some cultures after "slow" freezing in a dry ice-alcohol mixture at -78°C , but none was seen after "rapid" freezing at the lower temperatures. In the present study, the freezing rate, that is the time required for freezing to take place, was determined essentially by the temperature of the freezing agent. Such a direct relationship between freezing rates and freezing temperatures has been shown to exist (TAYLOR and GERSTNER, 1955). However, it is possible to alter and even reverse this direct relationship, for example by freezing at -198°C but either insulating the tissue or lowering the temperature slowly so that freezing would occur gradually and only after a long interval. It is difficult therefore to state with certainty whether the more favourable results at -78°C in our experiments were due to the slower freezing rate of the dry ice-alcohol mixture or to its higher freezing temperature. Preliminary experiments now under way to resolve this point indicate that the rate of freezing, and not the freezing temperature, is the critical factor, and that apparently the lowest freezing temperature can produce successful results if the

freezing time is lengthened. This would be a more favourable finding since metabolic changes are significantly less at -198°C than at -78°C (BECQUEREL, 1951). The greater preservative benefits of a slow freezing rate could then be combined with the storage advantages of very low freezing-temperatures for more effective and more prolonged storage of oral tissues.

Although our results may favour viable as opposed to non-viable teeth in implantation techniques, the use of living tissue can introduce a complicating factor. Vital adult teeth used in heterologous transplantations may induce undesirable reactions such as the immune or "homograft" reaction, causing loss of viability or even rejection of the transplant. The homograft reaction does not usually follow when foetal material is transplanted. The use of foetal tooth germs or buds may be an answer to this phase of the problem and satisfy the need for viability as well, or perhaps point the way to more successful preservation techniques for the storage and implantation of viable adult teeth.

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FIG. 2. Untreated, non-frozen, foetal, rat incisor tooth germ control. Living culture, unstained; grown *in vitro* for 5 days.

FIG. 3. 30% glycerol pretreated, non-frozen, foetal, rat molar tooth germ. Living culture, unstained; 7 days *in vitro*.

FIG. 4. 20% ethylene glycol pretreated, non-frozen, foetal, rat incisor tooth germ. Living culture, unstained; 5 days *in vitro*.

FIG. 5. Non-pretreated, foetal, rat molar tooth germ, frozen in liquid nitrogen (-198°C), cultured *in vitro* for 18 days. No cells have appeared in the culture.

FIG. 6. Non-pretreated, foetal, rat molar tooth germ, frozen in dry ice-ethyl alcohol (-78°C), cultured *in vitro* for 12 days. A few cells have appeared in the culture.

FIG. 7. 30% glycerol-pretreated, foetal, rat molar tooth germ, frozen in dry ice-ethyl alcohol (-78°C). Living culture, unstained; 6 days *in vitro*.

FIG. 8. 30% glycerol-pretreated, foetal, rat molar tooth germ, frozen in isopentane (-140°C). Living culture, unstained; 9 days *in vitro*.

FIG. 9. 30% glycerol-pretreated, foetal, rat molar tooth germ, frozen in liquid nitrogen (-198°C). Living culture, unstained; 8 days *in vitro*.

PRESERVATION AND STORAGE OF ORAL TISSUES—I

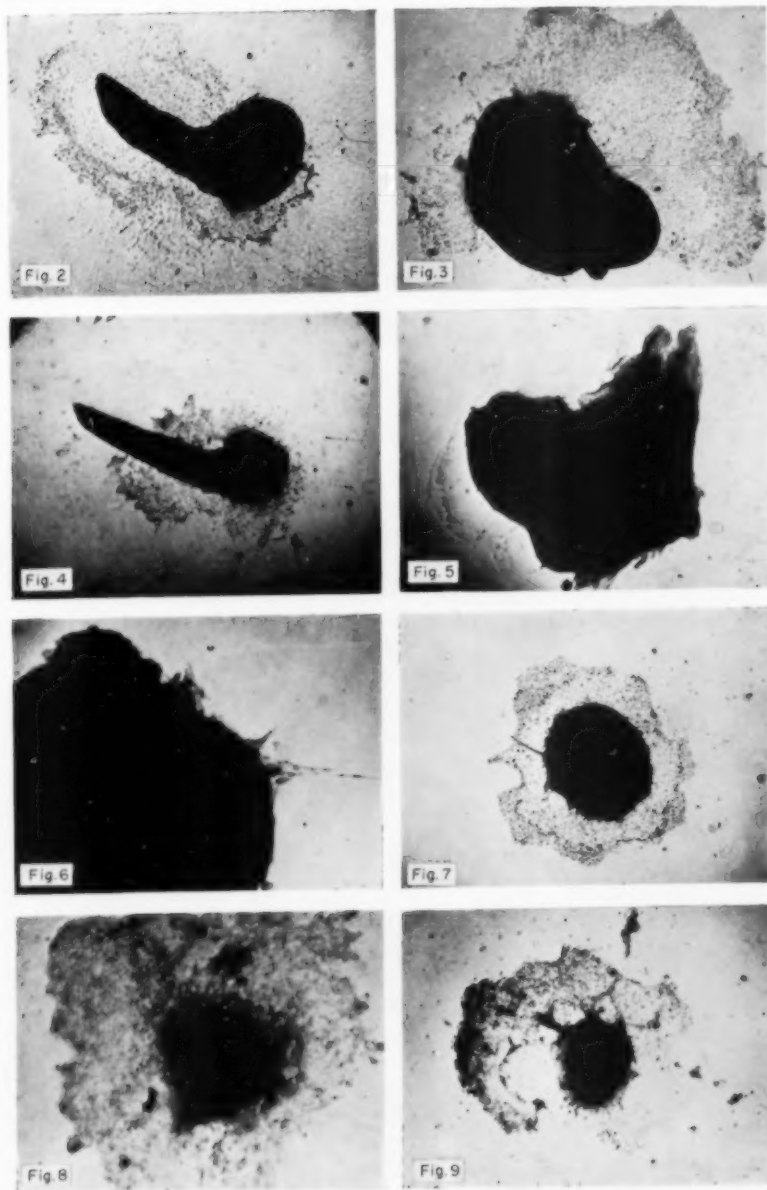


PLATE 1

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CITRATE IN MINERALIZED TISSUES—I

CITRATE IN HUMAN DENTINE

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Abstract—Two different methods of obtaining citrate from powdered dentine resulted in fractions both of which contained peptide. The peptide contained cystine, lysine, arginine, serine, glycine, glutamic acid, alanine, valine, leucine and tyrosine. No hydroxyproline and only traces of proline could be detected; it seems unlikely therefore that the peptide was a fragment of a collagen molecule.

The evidence obtained suggests that the citrate-peptide complex was released from dentine by cold dilute hydrochloric acid, and that it resisted mild alkaline hydrolysis with ethylene diamine at 120°C. None of the fractions contained detectable amounts of calcium.

DENTINE contains about 0.9 per cent of citrate (FREE, 1943; ZIPKIN and PIEZ, 1950; STACK, 1951), of which some 5 per cent can be removed by extracting powdered dentine with distilled water at room temperature, and a further 10 per cent by extraction with boiling water. These observations suggest that the citrate does not exist solely as the free calcium salt, the solubility of which, though low, should be high enough to permit its total extraction with water.

Little is known of the function of citrate in mineralized tissues, and the object of this preliminary investigation was to seek information concerning the nature of the citrate in dentine. Some evidence has been obtained which suggests that the citrate is associated with a peptide.

EXPERIMENTAL

Preparation of dentine

Freshly extracted human teeth were freed from fragments of soft tissue and adventitious material by the use of a dental bur. The teeth were cut at the cemento-enamel junction and the coronal portions discarded. The root canals were ground out and the cementum removed with a dental bur. The roots were then pulverized in a percussion mortar to a powder fine enough to pass through a 60-mesh sieve. All the results refer therefore to powdered root dentine.

Analytical methods

Nitrogen determinations were carried out by the micro-Kjeldahl technique (MARKHAM, 1942). Calcium was determined by its precipitation as oxalate and subsequent titration with 0.01N KMnO_4 .

Citrate analysis The method used was a modification of that described by HESS and WHITE (1955). Samples (4 ml) of solutions containing 0.1–1.0 mg of citric acid

were placed in graduated stoppered cylinders and 4 ml of 27N H_2SO_4 were added. The mixture was cooled to room temperature and 4 ml of the bromide-bromate-vanadate reagent added, the tubes were stoppered and allowed to stand for 45 min. To each tube, 2 ml of 20% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were added and the mixture allowed to stand for 10 min. Any excess bromine present was removed by the use of a suction pump. Six ml of *n*-heptane were now added and the mixture shaken for 90 sec to extract the pentabromoacetone from the aqueous layer. The two phases separated easily except when dissolved protein was present in the original sample. In these circumstances the layers were separated by centrifugation. Samples (5 ml) of the heptane layer were pipetted into tubes containing a little anhydrous Na_2SO_4 and allowed to stand for 2 min. The contents of the tubes were transferred to separating funnels containing 10 ml of freshly prepared 2% (w/v) Na_2S solution. The funnels were shaken for 30 sec, the layers allowed to separate, and the lower layer run directly into 1 cm cells and the absorption measured in a "Spekker" photoelectric absorptiometer, using an Ilford 601 violet filter. A calibration curve corresponding to 0.1–1.0 mg citric acid was prepared using samples of a standard solution of citric acid (analytical grade reagent). All analytical figures for citrate are calculated as citric acid.

Total citrate in dentine. Samples (approximately 1 g each) of powdered dentine were demineralized either by treating with an excess of N HCl for 24 hr or by shaking with an excess of a solution (10% w/v) of ethylene diamine tetra-acetate (EDTA) at pH 7.4 for 48 hr. In both cases the citrate went into solution and none could be detected in the demineralized residues. The quantities of citrate determined in the solutions, in each instance, corresponded with a dry dentine content of 0.87 per cent of citric acid.

Treatment of dentine with water

Powdered dentine (0.415 g) was shaken in an automatic shaker with 40 ml of distilled water (pH 6.5) for 180 hr. The water extract was evaporated to small bulk and was found to contain 0.2 mg of citrate which is about 5 per cent of the total citrate present in the dentine. A further sample (0.37 g) was treated with boiling water for 6 hr when about 7 per cent of the original citrate present was dissolved.

Treatment of dentine with ethylene glycol containing 3 per cent (w/v) of KOH

When the organic matter of dentine was removed by refluxing powdered dentine for 24 hr with ethylene glycol containing 3 per cent of KOH the washed residue contained no detectable citrate.

Treatment of dentine with ethylene diamine

A sample (1 g) of powdered dentine was first extracted for 48 hr in a Soxhlet extractor with ethylene diamine containing 14 per cent (v/v) of water. These proportions of the diamine and water gave a mixture of constant boiling point (118–120°C). The temperature in the extraction chamber, which was unjacketed, was about 80°C. The treated dentine was then extracted for 6 hr with ethanol to remove

excess ethylene diamine, and dried at 100°C. The resultant "anorganic" dentine contained 0.9 per cent of nitrogen and 1.2 per cent of citrate. This treatment had removed most of the nitrogenous material but none of the citrate. The value of 1.2 per cent of citrate in the extracted dentine corresponded to a value of approximately 0.9 per cent for whole dentine.

When a sample (0.368 g) of this "anorganic" dentine was shaken with 50 ml of cold distilled water for 36 hr, 1.6 mg of citrate was dissolved, this corresponded to 37 per cent of the total citrate in the sample. A further sample (0.311 g) of "anorganic" dentine was extracted for 18 hr with 50 ml of boiling water; this treatment dissolved 70 per cent of the total citrate. The residue was analysed and found to contain the balance of approximately 30 per cent of the citrate.

It can be concluded, therefore, that the alkaline hydrolytic removal of the dentinal protein with hot ethylene diamine rendered the citrate fraction more easily extractable with distilled water.

Citrate fraction obtained by extracting "anorganic" dentine with water

A sample (40 g) of "anorganic" dentine prepared as described above was extracted with boiling water for 4 hr. The solids were removed by filtration and the filtrate was evaporated to dryness under reduced pressure at 40°C. The residue remaining after evaporation contained about 65 per cent of the citrate originally present in the dentine. No calcium was detectable in the residue, which was insoluble in ethanol.

The material was dissolved in 10 ml of 2N HCl and again evaporated to dryness under reduced pressure. The resulting residue was now partially soluble in ethanol and the insoluble portion which remained contained only a small amount of citrate. The ethanol-soluble fraction, containing most of the citrate, was subjected to paper chromatography using methyl ethyl ketone-acetic acid-water (6:1:1) and Whatman No. 1 paper. The chromatograms showed a long streak of ninhydrin-positive material, but there was no resolution of individual amino acids. A large diffuse area about halfway down the streak gave a pink colour when it was sprayed with dimethylamino-benzaldehyde. This area corresponded approximately with the position of an authentic sample of citrate. This colour reaction is not specific for citrate, but in view of the known citrate content of our fractions it was considered justifiable to identify this provisionally as a citrate area. This suggested that the ethanol-soluble fraction contained citrate and a peptide. Another sample was hydrolysed by refluxing for 3 hr with 6N HCl. Chromatograms now showed the presence of at least eight amino acids which were provisionally identified by comparison with authentic samples as cystine, lysine, arginine, serine, glycine, glutamic acid, alanine and leucine. Further chromatograms prepared from a basic solvent, propanol-ammonia-water (6:3:1) revealed the presence of a spot corresponding to valine. In the acid solvent this amino acid was apparently obscured by the relatively large amount of citrate present. At this stage it is not possible to be certain of the composition of the peptide and small quantities of other amino acids may be present, e.g. threonine, aspartic acid and traces of proline. Until quantitative analyses are carried out these observations on the composition of the peptide must be considered provisional. Citrate was still

identifiable and appeared as a whitish zone in the ninhydrin-sprayed chromatograms. This zone was bounded at the top and bottom with crescents of ninhydrin-positive material which did not correspond with any of the common amino acids.

A further sample of the ethanol-soluble fraction was now hydrolysed for 18 hr with 6N HCl. Chromatograms prepared from this 18 hr hydrolysate were similar to the 3 hr hydrolysate except for the appearance of a faint ninhydrin-positive spot corresponding with that of an authentic sample of tyrosine, and the ninhydrin-positive crescents associated with the citrate zone were reduced in intensity (see Fig. 1).

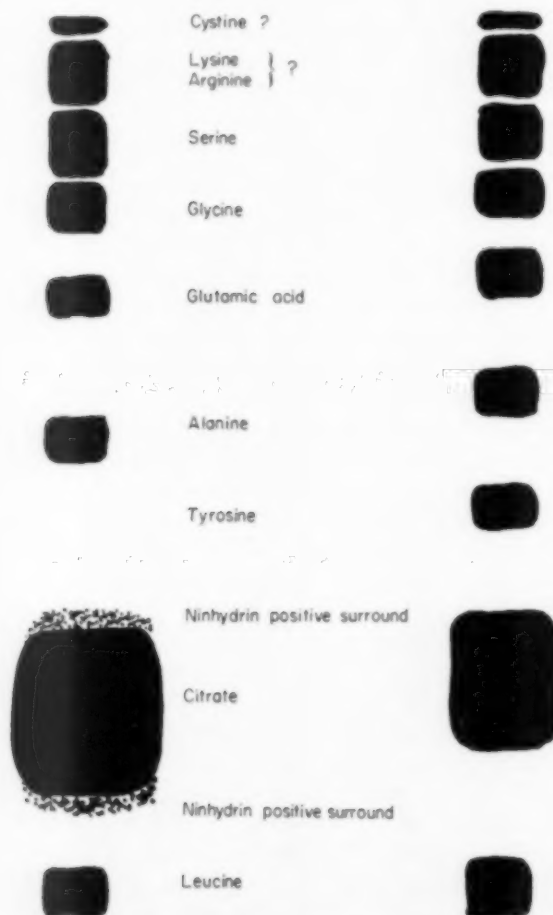


FIG. 1. Diagrammatic representation of the chromatograms obtained when the citrate/peptide complex was hydrolysed for 3 hr (left) and 18 hr (right). Note the appearance of a tyrosine spot after the longer period of hydrolysis.

These observations seemed to indicate that the citrate was associated in some way with a peptide and was perhaps attached to tyrosine. It was possible, of course, that the association was fortuitous; it was decided therefore to try other methods of isolating the citrate fraction to see if it was still associated with a peptide.

Citrate fraction obtained by demineralization of dentine with N HCl

A sample (50 g) of powdered dentine was treated with an excess (1 litre) of N HCl and the mixture stirred mechanically. Demineralization was complete in 24 hr, and the mixture was filtered. The filtrate contained all the citrate and about 5 per cent of the total nitrogen present in the original dentine. The acid filtrate was shaken with 20 g of powdered charcoal (Norite) for 30 min and then filtered. All the citrate was adsorbed on to the charcoal; it was then thoroughly washed with water and eluted six times with ammoniacal aqueous ethanol (10 vol NH_3 , 0.880 s.g.; 50 vol water; 40 vol ethanol). The eluates were combined and evaporated to dryness under reduced pressure. The residue contained about 15 per cent of the citrate originally present in the dentine, and was in a form which was insoluble in ethanol. On acidification with 2N HCl and evaporation to dryness the citrate became soluble in ethanol. Paper chromatograms of the ethanol extract again showed a streak of ninhydrin-positive material with a zone of citric acid situated halfway down the streak. Hydrolysis of the extract with 6N HCl for 3 hr followed by paper chromatography revealed the presence of a number of amino acids substantially the same as those identified after the hydrolysis of material obtained from the extraction of anorganic dentine. The citrate zone bounded by its accompanying ninhydrin-positive crescents was again present. More vigorous hydrolysis with 6N HCl for 18 hr produced a mixture, chromatograms of which showed reduced amounts of the ninhydrin-positive crescents, and a faint but identifiable tyrosine spot. This observation again suggested the association of citrate with a peptide.

Reprecipitation of the mineral fraction of the acid-soluble portion of dentine

A further sample (40 g) of powdered dentine was demineralized with hydrochloric acid as described above. The filtrate was made alkaline by the addition of an excess of ammonia (0.880 s.g.). The precipitate of dentine minerals so formed was collected by filtration, the filtrate contained no citrate. The precipitate was thoroughly washed with water, and the solids were collected and dried at 100°C. Analysis of these solids showed that they contained 95 per cent of the citrate originally present in the dentine. This solid fraction was redissolved in 2N HCl, care being taken to avoid an excess of acid. The solution was now treated with 10N H_2SO_4 until no further precipitation occurred. The precipitate was collected by filtration and washed three times with water. Analysis showed that all the citrate was present in the filtrate; none was carried down with the insoluble sulphates. The acid filtrate was now treated with charcoal and eluted as before. The eluates contained a peptide and citrate which on hydrolysis gave chromatograms similar to those described earlier.

Thus, differing methods of treatment of dentine resulted in the preparation of citrate fractions all of which contained a peptide of similar amino acid composition.

DISCUSSION

Of the total organic matter present in dentine nearly 5 per cent is citrate. It does not appear to be present as the simple calcium salt. The dissolution of the mineral phase of dentine either with acid or with EDTA resulted in the solution of the citrate fraction together with some soluble peptide. When the organic material was removed from dentine by boiling with ethylene glycol containing 3 per cent (w/v) of KOH the mineral residue did not contain any detectable citrate. When, however, dentine was extracted under milder conditions with ethylene diamine, most of the nitrogenous material was removed, but the citrate remained with the mineral phase. About 65-70 per cent of the citrate was now extractable with water. LOSEE and HURLEY (1956) reported that samples of anorganic bone and dentine which had been thoroughly washed with water contained 17.9 and 18.4 per cent of the original citrate, respectively. Our results confirm their observation that there is a portion of the citrate which is not easily rendered water-soluble by treatment with ethylene diamine.

The citrate fraction obtained from the ethylene diamine-treated dentine and that resulting from the acid dissolution of dentine were both associated with a similar peptide.

These results suggest that not all of the citrate is firmly bound either to the mineral phase or to the major collagenous portion of the protein matrix, but is associated with a peptide which is not likely to be a fragment of the collagen molecule. This peptide was extractable from dentine with cold dilute hydrochloric acid, but was not extracted by the treatment of dentine with boiling ethylene diamine. In addition it contained only traces of proline and no detectable hydroxyproline. It seems likely that the peptide is part of the acid-soluble dentinal protein described by LOSEE, LEOPOLD and HESS (1951) and also by STACK (1951).

The observation that the citrate fraction was rendered soluble in ethanol by treatment with cold dilute acid indicated that its carboxyl groups were not combined in a covalent linkage. It is of considerable interest that tyrosine was detectable only after prolonged hydrolysis, and that the event was accompanied by a reduction in intensity of the ninhydrin-positive crescents on the citrate spot on the chromatogram. It suggests that tyrosine and citrate may be linked in a reasonably stable manner. The possibility that they are combined in an ether linkage must be investigated. If, indeed, tyrosine and citrate are directly combined, it could only account for a portion of the citrate, since there does not appear to be enough tyrosine in dentine to combine with all the citrate.

The results so far do not enable any definite conclusions to be drawn concerning a possible function of citrate in dentine. It seems probable, however, that if some citrate is associated with a peptide its presence in a mineralized tissue is not entirely adventitious, as was suggested by ARMSTRONG and SINGER (1956) in their studies on bone. Citrate appears to be intimately involved with the metabolism of calcium and phosphorus. There is considerable evidence to show the influence of vitamin D, and calcium and phosphorus intake upon the citrate content of bone (STEENBOCK and BELLIN, 1953; CARLSSON and HOLLUNGER, 1954; NICOLAYSON and EEG-LARSEN, 1956).

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These results are of a preliminary nature; it is intended to gather more information concerning the peptide and the nature of its association with the citrate in dentine and in other mineralized tissues.

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THE INFECTIOUS AND TRANSMISSIBLE NATURE OF EXPERIMENTAL DENTAL CARIES. FINDINGS AND IMPLICATIONS

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Abstract—In both hamsters and Osborne-Mendel rats dental caries appears to be an infectious and transmissible disease which involves a penicillin-sensitive flora. The findings reported suggest that if this flora is present or is inoculated into young animals, rampant caries can be induced by a high-carbohydrate low-fat diet within 35 days. When low to negligible caries has been found in animals similarly fed, the flora presumably has not been present in sufficient quantity to be pathogenic. Caries activity was markedly reduced in hamsters and rats after the penicillin-sensitive flora had been depressed prior to feeding the test diet. Hamsters whose flora had been depressed, in some cases, produced several generations of progeny with negligible activity. Marked differences in activity apparently result from alterations or variables in the flora which animals acquire, transmit, and develop during the course of an experiment. Thus, a number of interpretations, which have been made to explain differences in caries activity observed in past studies, must be reconsidered in terms of variations possibly induced in the microbial flora.

This paper reports experiments which demonstrate the infectious and transmissible nature of dental caries in hamsters and in Osborne-Mendel rats, opens for reconsideration the nature of the animal-microflora diet relationship, and may explain some of the divergent findings reported over the past 35 years.

In studies of experimental dental caries, variability has often been considerable between animals in the same laboratory and so wide between animals in different laboratories that results have not been reproducible (ARNOLD, 1942; MCCLURE, 1945; GUSTAFSON, STELLING and BRUNIUS, 1952; KEYES, 1954; JOHANSEN and KEYES, 1955; STEPHAN and HARRIS, 1955; HUNT, HOPPERT and ROSEN, 1955; MITCHELL and JOHNSON, 1957). The factors most frequently investigated to account for these differences in "susceptibility" include those concerning bacteria, genetics, hormones and nutrition (FITZGERALD, 1955; HUNT *et al.*, 1955; MCCLURE, 1955; ORLAND, 1955; SHAFER and MUHLER, 1955).

When evidence was found that "susceptibility" to caries in hamsters showed intra-litter tendencies (ARNOLD, 1942; HODGE, JOHANSEN and HEIN, 1953), efforts were directed toward the breeding of lines with a consistent caries history (HODGE, JOHANSEN and HEIN, 1955; JOHANSEN and HODGE, 1956). Our own results from this approach were not productive, and several observations prompted the presently reported studies related to transmissible factors. (1) A number of attempts to breed caries-active litters indicated that "susceptibility" was not influenced by the caries

history of the sire, either in the first generation or in the second, when fathers were back-crossed with daughters. The activity of litters seemed to coincide with that of the mother. (2) Animals which had remained essentially free of caries for extended periods on a caries-test diet had suddenly produced active litters (KEYES, 1959), and this change in susceptibility occurred more generally than genetic mutation would explain. (3) Susceptibility occasionally varied widely between different cages; animals mixed together in one cage developed caries while litter-mates kept together in other cages showed negligible activity.

PROCEDURES AND RESULTS

The methods employed to induce rampant caries in hamsters within a period of 5-6 weeks have been described in detail (KEYES, 1959) and were essentially the same as those used in the experiments to be reported. The caries-test diet was fed to young weanlings approximately 21 days old and 30 g in weight. [The diet was composed of confectionery sugar 59 per cent; skim milk powder 27 per cent; whole wheat flour 6 per cent; alfalfa powder 3 per cent; liver powder (1:20, Wilson) 2 per cent; whole liver powder 2 per cent; salt mixture 1 per cent. (KEYES, 1959).] Distilled water was always available and supplements of fresh apple, carrot and kale were provided once a week. In general, animals were kept on this regimen for 35 or 42 days and then placed on a stock diet of finely ground laboratory chow for 21 days. [Purina Laboratory Chow (meal form) Ralston Purina Co., St. Louis, Mo., and BB Laboratory Rabbit Diet, Maritime Milling Co., Buffalo, New York.] Then they were examined and finally scored by methods previously described (KEYES, 1944, 1959). Six experiments are reported and discussed below.

The first illustrates the transmissible nature of caries in hamsters.

The second suggests that the principal source of the cariogenic flora in weanlings may come from maternal faeces.

The third demonstrates that the flora can be inoculated and confirms findings in Experiment 1.

The fourth shows the effect of penicillin and erythromycin on caries activity and on that of a subsequent generation.

The fifth shows the effect on offspring of depressing the penicillin sensitive flora of mothers.

The sixth, employing Osborne-Mendel rats, shows the effect on caries activity of depressing the penicillin-sensitive flora during the pre-weanling period and illustrates transmissibility in this species.

Experiment 1

Two tests were undertaken to assess the possibility that dental caries might be a transmissible disease in hamsters. The first assay used a sample of piebald animals; and the second, a group of albinos. The weanlings from each of these two strains were divided so that, while receiving the caries-test diet, some were caged in small groupings by themselves and others were kept with caries-active animals.

Thus, 27 male and 10 female piebald hamsters, which were supplied by the National Institutes of Health animal production station and which averaged 30 g in weight, were divided as equally as possible. (At this point in the investigation, it was assumed that these animals might not be highly infected with the "cariogenic" flora. This possibility proved to be correct but was not known definitely when the experiment was started.) The 18 animals which comprised the control group were distributed into cages, 4-5 per cage, and were not allowed contact with other animals in the colony. The remaining 19 animals were placed in cages with hamsters known to be caries-active. The number of animals per cage was again 4-5. The caries-test diet and distilled water were available to all animals *ad libitum*.

TABLE 1. CARIES ACTIVITY IN PIEBALD AND ALBINO HAMSTERS. HALF OF EACH GROUP WAS CAGED WITH CARIES ACTIVE ANIMALS

Groups	No. of animals	Percentage of carious teeth	Range of scores	Average scores	Average maxillary molar scores		
					1st	2nd	3rd
Piebald							
Controls	18	42.0	0-15	3.5	0.1	1.4	1.4
Caged with actives	19	94.3	6-216	74.8	3.2	27.3	18.0
Albino							
Controls	17	29.9	1-21	9.6	0.01	9.3	0.1
Caged with actives	12	83.3	22-105	57.2	1.2	32.9	4.6

After 35 days on the diet the animals exposed to caries-active hamsters had obvious cavitation, while those not so exposed were almost free of lesions. To demonstrate the difference in caries activity between the control and mixed groups more clearly, the experimental period was extended to 56 days and final scores were assigned after the animals had completed an additional 21 day period on the stock diet. Results are reported in Table 1. Typical jaws from a control and contaminated group of hamsters are shown in Figs. 1 and 2.

The second assay, using a series of albino hamsters, repeated in principle the test with piebald animals and kept the experimental conditions from birth on as close as possible to those usually employed (KEYES, 1959). Thus the albinos were born and raised in the environment of our laboratory, and as a consequence they were started on the experimental regimen when slightly younger than the piebalds. Caries-inactive females which, for the most part, were sent from the N.I.H. animal production station while in a late stage of pregnancy produced the weanlings used. Again, it was hoped that the animals so raised might not be "infected", because young albino hamsters previously supplied by the production station had developed very little caries when tested (KEYES, 1959).

Twenty-nine albino hamsters were obtained from the albino females mentioned above. Seventeen were caged in groupings by themselves, and 12 were mixed among other caries active animals in the colony. The experimental period included the usual 35 days on the caries-test diet and 21 days on finely ground laboratory chows (KEYES, 1959). The findings from this study were in agreement with those previously described and are also presented in Table 1.

To illustrate further the low caries activity that may be found in "non-infected" hamsters, three additional albino litters were raised in our laboratory from females newly acquired from the production station. A total of 23 weanlings was produced. At 20 days of age these were caged in small groups and fed the caries-test diet for 64 days plus the usual 21 days on stock chows. Minimal evidence of caries activity was detected while animals were on the test diet. The final scores per litter averaged 9.8, 26.3 and 19. By contrast, the usual caries-active litter develops rampant cavitation in 35 days which usually involves all teeth (KEYES, 1959).

Experiment 2

This was undertaken to determine whether animals born of non-infectious females would develop caries following an inoculation, during the suckling period, of faecal material from caries-active animals. It had become evident that some factor, presumably a microbic flora, had to be acquired early in the life of the animals,

TABLE 2. THE EFFECT OF INOCULATION OF FAECES INTO THE MOUTHS OF WEANLING HAMSTERS BETWEEN 7 AND 21 DAYS AFTER BIRTH

Animal Numbers	Caries score	
	After 35 days	Final
Litter NW	Inoculated	
86	29	84
87	47	142
88	28	64
	Non-Inoculated	
89	25	47
90	0	5
91	0	4
92	0	3
Litter PJ	Inoculated	
111	37	112
112	13	79
113	28	77
	Non-Inoculated	
114	0	Not determined
115	0	due to accidental
116	0	death of group

such as during the suckling period, to induce the high level of activity observed in previous studies (KEYES, 1959). That this factor was not attributable to the diet or the general environment was suggested by previous findings and demonstrated in Experiment 1.

Several pregnant golden and albino females were obtained from the N.I.H. animal production station and assumed to be "uninfected", i.e. not carriers of the "cariogenic" flora. Seven days after birth half of each litter was marked with methylene blue, and these animals were inoculated orally for the next 14 days with a smear of a few milligrammes of faecal material from caries-active animals. Upon weaning at 21 days of age the inoculated and non-inoculated animals were separated, caged and tested.

All inoculated animals became caries-active; indeed, in the preliminary tests all of the weanlings developed lesions, probably as a result of cross-contamination. This result, in itself, was different from what had been found in non-infected litters produced by females in Experiment 1. Because it seemed impractical to follow this approach further, other studies were undertaken. However, two litters showed a clear-cut difference between inoculated and non-inoculated animals. Data in Table 2 show that active caries developed in those animals which received the oral smears, while untreated litter-mates developed little or negligible activity. While the study was not definitive, four points were suggested: (1) maternal faeces might be the principal source of the "cariogenic" flora acquired during the suckling period; (2) the flora acquired prior to weaning and experimental testing can have a profound effect on the end results; (3) the influence of nutritional status during pregnancy, lactation and early weanling period cannot be assessed by any of the routine methods used to date, and (4) faecal material might be used as a crude inoculum in tests of the feasibility of future inoculation studies.

Experiment 3

In this study an attempt was made to induce caries activity by an inoculation of faeces from caries-active hamsters. Faecal material was used as either an intra-oral smear or as a contaminator of the drinking water. Five successive series of 9-12 albino hamsters from the N.I.H. animal production station were assessed according to the procedure indicated by Table 3. One third of each group was caged by itself and not exposed to a source of gross contamination. One third was exposed to caries-active animals in the various ratios indicated, and the other third was inoculated with faeces. The drinking water was contaminated by mixing freshly collected faecal pellets from caries-active animals with distilled water, four pellets to 100 ml. All the animals, except Series 5, consumed the caries-test diet for at least 42 days before final evaluation.

Findings are also reported in Table 3. In control animals caries activity was negligible, and only one animal in the third series had a score of 30. All animals exposed to those which were caries-active developed caries, and many had small visible lesions within 2 weeks. Indeed, some animals became so active that they were continued on the experimental diet and used for infecting a succeeding sample of

animals (Table 3, Series 2). Thus, activity was passed to a new group by using animals activated by a previous test. In the fourth and fifth series the animals were older than those previously used. In Series 4, at the end of 42 days the 4 hamsters which were exposed to one caries-active animal showed definite activity but to a less advanced degree than litter-mates which received oral smears. To demonstrate that the activity of these animals would approach that in the other groups, they were continued on the experimental diet, along with control litter-mates, for 6 additional days. In the fifth series the experimental period was inadvertently terminated after 35 days on the test ration. Consequently, the scores were lower but followed the trend of previous tests.

TABLE 3. THE INDUCTION OF CARIES ACTIVITY IN ALBINO HAMSTERS BY MEANS OF EXPOSURE TO SUSCEPTIBLE ANIMALS OR BY THE ORAL INOCULATION OF FAECES FROM CARIES-ACTIVE ANIMALS

Series	Total caries scores of individual animals		
	Controls not mixed	Mixed with caries-active animals	Inoculated with faecal material
Age 24 days 1. Average weight 35 g Test period 42 days	3, 6, 5	75, 120, 153 (ratio 1:3 or 4 susceptible animals for 20 days)	68, 89, 114 (faeces in water for first 20 days)
Age 22 days 2. Average weight 32 g Test period 42 days	0, 0, 0	102, 100*, 100* (ratio 3:1 susceptible animals for 21 days)	79, 99, 133 (inoculated as above)
Age 23 days 3. Average weight 29 g Test period 42 days	8, 11, 30	82, 86, 100 (ratio 3:1 susceptible animals for 22 days)	76, 112, 126 (inoculated as above)
Age 25 days 4. Average weight 43 g Test period 48 days	0, 1, 4, 10	71, 84, 33, 21 (ratio 4:1 susceptible animals for 21 days)	46, 65, 75, 82 (faecal smear for first five days of 42 day test period)
Age 26 days 5. Average weight 42 g Test period 35 days	0, 0, 0	37, 63, +† (ratio 2:2 susceptible animals)	30, 43, 56 (faecal smear for first 7 days)

* Caries was so active in these animals they were kept on experimental diet and used as source of contamination in other trials.

† Animal killed because of diarrhoea. Lesions were not scored because experimental period was less than 35 days.

The findings of this experiment demonstrated that (1) the caries activity could be induced by an inoculum and opened the way for inoculation studies with specific organisms. (2) The findings reported in Experiment 1 were again confirmed in regard

to transmissibility between animals of the same species. (3) Faecal material was shown to be a possible source of the "cariogenic" flora, a finding in agreement with the observation in Experiment 2.

Eight of the inoculated females used in this experiment were bred to determine whether they would produce caries-active litters. Litters from 3 non-inoculated females in the control group were also tested. This side study was undertaken because several previous, unreported, attempts to produce caries-active litters had been largely unsuccessful when production-station females were bred after they had received the test diet alone for 42 or 56 days. It was of interest, therefore, to find that the 8 females mentioned above, which received the test diet for 42 days and which were activated either by exposure or by inoculation, produced litters whose scores averaged 198, 178, 159, 159, 98, 86, 86 and 20. The 3 control females which were not intentionally infected produced litters with average scores of 88, 32, and 10. Thus, the high activity in the litters from inoculated females was in marked contrast to the low activity previously found, again seen in two of the control litters, and also to be reported in litters in Experiments 4 and 5.

These findings offered an explanation for the high caries activity first observed in litters born of albino hamsters which came from the N.I.H. animal production station (KEYES, 1959). It was probably due to a cariogenic flora which had been acquired or enhanced while animals were on the over 100 day exposure to the test diet previously used. A 42 or 56 day feeding of the test diet alone may not, in itself, sufficiently enhance a flora with a high "cariogenic" potential.

Experiment 4

This study was undertaken to observe the effect on caries activity of antibiotic added to the caries-test diet. In addition, females which were caries-inactive as a result of this antibiotic treatment were bred, and their litters were assayed to determine if activity would revert to the low level described for litters which apparently do not have the "cariogenic" flora.

From a line of albino hamsters which had shown consistent caries activity for three generations, a fourth generation litter of 3 females and 4 males was selected for study. Upon weaning, 2 females and 2 males were fed for 35 days the caries-test diet containing either 100 mg of penicillin ("Compenamine", Commercial Solvents Corporation) or 100 mg of erythromycin per kilogramme. As indicated in Table 4, at the end of the experiment the 4 animals which received the antibiotics were free of caries, while the teeth of untreated control litter-mates showed typical activity, with scores of 92, 85 and 75. A short time after placement on the stock diet, the caries-free female (No. 678), which had received erythromycin, and the untreated control sister (No. 676) produced litters which were assessed for caries activity by methods previously described. The weanlings from female No. 678 were divided: while on the test diet, 3 were kept by themselves and 2 were placed with other caries-active animals in the colony. The litter from the untreated, caries-active female No. 676 was placed in a cage by itself and not exposed to other animals in the colony.

TABLE 4. CARIES SCORES IN THREE GENERATIONS OF HAMSTERS FED AND TREATED AS INDICATED IN COLUMNS THREE AND FIVE

Generation	Animal no.	Caries-test diet	Scores	Caging
f_4 (♀W217 × ♂W163)	♂672	only	92	Together
	♂673	only	85	Together
	♀676	only	75	Together
	♂674	+ Pencillin	0	Together
	♀677	+ Penicillin	0	Together
	♂675	+ Erythromycin	0	Together
	♀678	+ Erythromycin	0	Together
f_5 (♀W676 × ♂W674)	♂760	only	135	Together
	♂761	only	120	Together
	♂762	only	126	Together
	♂763	only	119	Together
(♀W678 × ♂W675)	♂732	only	188	With susceptible
	♀735	only	176	
	♂733	only	0	Together
	♀734	only	0	Together
	♀736	only	0	Together
(♀W677 × ♂W674)	♂791	only	82	Together
	♂792	only	175	Together
	♀793	only	106	Together
	♀794	only	56	Together
f_6 (♀W735 × ♂W732)	♂808	only	183	Together
	♂809	only	195	Together
	♂810	only	208	Together
	♀811	only	80	Together
(♀W794 × ♂W792)	♂848	only	143	Together
	♂849	only	141	Together
	♀850	only	100	Together
	♀851	only	75	Together

As indicated in Table 4, the weanlings from female No. 678, which had previously received erythromycin, were caries-free when caged by themselves. However, littermates (No. 732 and No. 735) which were exposed to caries-active animals developed rampant caries. Weanlings from female No. 676, which was not fed on antibiotics, developed typical rampant caries. From female No. 677, a litter was finally produced 112 days after termination of the penicillin-treated test ration. Caries activity in this litter was not less than that usually found in active lines. There are several possible

reasons for this activity, e.g. (1) too long a period may have elapsed between the time of removal from the antibiotic and the birth of the litter so that the flora re-established itself in the alimentary canal of the mother animal. (2) The penicillin was effective in reducing the "cariogenic" flora within the mouth but may not have depressed that in the alimentary canal as a whole. (3) Contamination of the litter may have occurred. In order to show conclusively that this line of hamsters was "caries-susceptible", an assessment was made of two sixth-generation litters from caries-active females Nos. 735 and 794. The data in Table 4 show that these litters were highly caries-active although their grandmothers had been caries-free.

This experiment showed that penicillin or erythromycin will suppress caries activity when present in the caries-test diet at the level used. Females in which the "cariogenic" flora has been depressed may, in some instances, deliver litters which will continue to show negligible caries activity. Again it was demonstrated that animals which do not carry the cariogenic flora can acquire it by exposure to caries-active animals, a finding in agreement with observations in Experiments 1 and 3.

Experiment 5

The purpose of this study was: (1) to observe the degree of caries activity in litters produced by females whose penicillin-sensitive flora had been depressed during pregnancy and the first week of lactation; (2) to determine whether litters from active lines would show a change in caries activity if their dams never consumed the caries-test diet but instead consumed only the stock ration throughout their lives; (3) to assess caries activity in litters from females which were inactive as a result of the previous feeding of antibiotic to their mothers.

From several litters with an established history of high caries activity females were selected for future breeding and allocated to three groups. Brothers were used as sires.

Group A. Litters of this group were from dams fed the caries-test diet and the stock ration. The weanlings in this sample were born of females which, prior to mating, had received the caries-test diet for 35 days and which as a result developed active carious lesions. Thus, this group of animals was raised according to previously reported routine (KEYES, 1959).

Group B. Litters of this group were from dams fed the caries-test diet and then the stock ration plus antibiotic. These dams had had active caries as had their mothers before them. (The caries scores in the female breeders allocated to Groups A and B ranged between 53 and 130 and averaged 95). When the females in this group were placed on the stock ration for breeding, penicillin ("Compenamine P-92", Commercial Solvents Corporation) was added at a level of 100 units/g of diet. The first females to be treated with the antibiotic in the stock diet received it immediately after termination of the caries-test ration. Because they seemed to have difficulty in adjusting to this regimen, subsequent animals were given untreated stock for one week before the antibiotic was introduced.

The penicillin-treated diet was fed to most of the females up to and including conception and pregnancy, and this ration was continued during the first 7 days of lactation. It was then withdrawn to prevent the young from having direct access to the antibiotic, and regular stock diet was then substituted.

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Group C. These litters were from dams which had been maintained continuously on the stock diet and which, never having consumed the caries-test diet, were free of carious lesions. However, the grandmothers of these litters had developed caries when tested.

All litters raised in Groups A, B, and C were evaluated after the usual 35 day exposure to the caries-test diet and 21 days on stock ration. In Group A five litters were assessed. This group of litters, however, can be considered comparable to those routinely studied for caries activity in the colony. In Group B, considerable difficulty was experienced with the litters born of mothers which received the penicillin. The mortality of the newly born animals was discouragingly high and seemed to be associated with poor lactation. However, despite a number of failures, a sample of litters was obtained from the females which received the antibiotic. The smaller litter size in this group does not affect the interpretation of the findings, as under present experimental conditions no correlation has been found between size of litter and degree of caries activity (KEYES, 1959). In Group C, eleven litters were produced by the females which were never fed the caries-test diet but which received the stock diet continuously after birth. In Table 5 the average caries scores and the average number of carious molars per animal are presented for each of the litters.

The results were as follows: *Group A* litters (from dams subjected to the usual procedure of caries-testing and breeding) developed caries activity which was typical of that seen in similarly treated animals in the colony. In *Group B* ten litters (from dams fed penicillin in the stock diet) showed negligible activity. Thus the transmission

TABLE 5. THE AVERAGE SCORES AND MOLARS AFFECTED IN LITTERS FROM DAMS IN GROUPS A, B AND C

Group A Dams on test diet and stock			Group B Dams on test diet and stock plus penicillin			Group C Dams fed stock diet only		
Litter	Average score	Average molars	Litter	Average score	Average molars	Litter	Average score	Average molars
*			*			*		
MK (5)	126	9.5	NX (4)	0.3	1.0	LT (3)	131	11.7
MS (4)	101	12.0	PE (3)	0.0	0.0	MH (6)	78	10.0
MY (5)	133	12.0	OL (7)	3.0	2.4	LO (6)	102	9.8
NK (3)	138	11.0	QK (1)	0.0	0.0	NF (8)	122	12.0
MU (6)	164	11.3	PB (4)	0.5	2.8	LP (4)	137	12.0
			RN (2)	0.0	0.0	MG (6)	116	12.0
			OU (1)	0.0	0.0	LV (3)	126	12.0
			NN (5)	3.6	7.6	MO (3)	137	11.0
			NJ (4)	5.7	8.0	MT (7)	16	7.4
			NV (3)	0.0	0.0	MM (8)	89	11.1
			Exceptions			ME (6)	90	11.5
			OS (5)	41.0	9.4			
			MB (7)	141.0	11.7			

* Numbers in parentheses indicate size of litter.

of dental caries activity from dam to offspring was disrupted by depressing the penicillin-sensitive flora of the mothers. In two of the twelve litters assessed (OS and MB) the degree of activity observed was comparable to the controls (Group A). This finding may represent an experimental variable, or it may be the result of a technical error in handling of the animals. *Group C* litters (from dams which received stock diet only) were as caries active as those in *Group A*.

The findings of this experiment showed that depression of the penicillin-sensitive flora in female breeders resulted in an almost complete loss of caries activity in offspring. All the breeders in this group had had active caries and had been exposed to a strenuous, and somewhat deficient, caries-test diet for 35 days prior to commencement of the stock diet and mating. All *Group C* litters were highly active, although the dams in this group were themselves caries-free and had never been subjected to the stress of the caries-testing regimen. Thus, it appears that these dams continued to carry the "cariogenic" flora and infected their offspring in whom it was then activated by the test diet. The high caries activity in these latter litters cannot be considered the result of pre-natal dietary deficiencies imposed by a nutritionally deficient caries-test ration. It should also be emphasized that during the suckling and early weanling period the diet was as complete as possible in the light of present knowledge.

Additional observations were made along the lines described in Experiment 5, in which caries-inactive females were bred to determine the level of activity in offspring. Again no further antibiotic was used following the first depression of the flora in the "original" female parent. Thus, several litters were raised from inactive females found in *Group B*.

One female from litter NX produced offspring which were highly caries active when assessed. Three females from the inactive litter PB produced litters which remained caries free while on the test diet; and three subsequent generations from one of these litters have remained completely inactive, i.e. following the original depression of the flora in one female, five generations have remained inactive to date. From litter NV one female produced a litter with high activity, whilst a sister produced a litter with low activity. These findings suggest that, in certain cases, if the cariogenic flora be sufficiently depressed and not reintroduced, litters will remain inactive through several generations despite the fact that they are exposed to the experimental diet for a 35 day test period.

Experiment 6

Osborne-Mendel rats were used in this experiment which was undertaken (1) to determine if caries activity in this species would be altered by depressing the penicillin-sensitive flora during the suckling period, i.e. before the experimental diet; and (2) to determine if the disease would show evidence of transmissibility demonstrated in hamsters.

Six pregnant Osborne-Mendel rats were obtained from the N.I.H. animal production station. This strain was selected because, in approximately 100 animals used for other studies, all developed a wide variety of carious lesions when fed the

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same caries-test diet as used in hamster studies. The type and distribution of lesions on this ration have been previously described (KEYES, 1958a).

Two of the females were fed regular stock diet during the last few days of pregnancy and throughout the suckling period. The weanlings from these litters were used as controls. Another 4 females and their litters received the same stock ration containing 100 units of penicillin per gram. This diet was fed during the last days of pregnancy and until the young were weaned. Eight young rats were removed from the females which received the penicillin and placed with control animals as follows: 10-12 days after birth 4 nursing rats were added to the control litters and, at weaning, were separated from the controls and tested in a separate cage by themselves. The other 4 animals from the antibiotic treated litters were caged with control weanlings at the time the experimental diet was started, i.e. when 25 days of age. The results from these eight animals have been combined in Table 6, as the scores of caries activity were not sufficiently different to require separate listing.

TABLE 6. AVERAGE DENTAL CARIES SCORES IN GROUPS OF OSBORNE-MENDEL RATS FED STOCK DIET ONLY (CONTROLS), STOCK WITH PENICILLIN (ANTIBIOTICS), AND IN THE ANTIBIOTIC FED GROUP CAGED WITH CONTROLS

Groups	Types of lesions									
	Bucco-lingual			Major sulcal			Approximal			Minor sulcal
Control	No. of lesions	Enamel score	Dentine score (D _s)	No. of lesions	Enamel score	Dentine score (D _s)	No. of lesions	Enamel score	Dentine score (D _s)	No. of lesions
Controls (11)*	10.0	23.4	7.3	18.3	27.4	16.9	5.9	5.6	1.5	6.6
Antibiotics (17)	0.6	1.2	0.3	11.8	15.4	4.3	0.5	0.5	0.0	0.0
Antibiotics caged with controls (8)	2.8	7.8	2.1	18.4	30.3	21.3	6.5	6.5	1.3	6.5

* Number of animals in parenthesis.

After 50 days on the experimental diet the animals were killed, the jaws were stained, hemi-sectioned and evaluated by methods previously described (KEYES, 1958b). Results are reported in Table 6 in which both maxillary and mandibular scores have been combined in each category of lesions. Photographs of representative jaws from each of the three groupings are shown in Fig. 3.

In the non-treated animals (controls) lesions were found on all surfaces of the teeth in a pattern typical for Osborne-Mendel rats fed this diet (KEYES, 1958a). All maxillary and mandibular molars were affected with either smooth-surface (bucco-lingual and approximal), sulcal (major and minor types) or both kinds of lesions. Many had penetrated well into the dentine as indicated by the dentine scores (D_s).

Caries activity was markedly reduced in animals fed penicillin during the suckling period (antibiotics). Smooth-surface activity was negligible, and no minor sulcal lesions were detected. In the major sulci, however, slight caries activity had developed, but the number of lesions in this category was smaller, and few had penetrated into the dentine as indicated by the low dentinal scores of 4.3. Indeed, no Osborne-Mendel rat fed this caries-test ration to date has been found with the little activity seen in this group. The possibility that these animals were inactive because of chance immunity or resistance is ruled out by the fact that litter-mates developed active caries when exposed to the control animals whose flora had not been depressed.

In those animals removed from the antibiotic, either during the suckling period or upon weaning, and placed in contact with controls, activity reached that of control animals in all areas except buccal surfaces, where it was somewhat less. This finding again illustrates the importance of assessing all types of lesions rather than primarily one type—a point previously discussed (KEYES, 1958b; KEYES and WHITE, 1959).

The findings in this experiment (1) suggest that the flora which the animals acquire before weaning and during the course of the experiment has an important influence on the incidence and size of lesions; (2) indicate that caries was less active in rats whose penicillin-sensitive flora had been depressed during the suckling and pre-weanling period; and (3) demonstrate that between susceptible Osborne-Mendel rats dental caries can be a transmissible disease.

DISCUSSION

The observations reported in this paper seem to explain some of the variable findings that have been observed in studies of experimental animal caries, and they suggest other interpretations for the results obtained in previous investigations. Because the relationship between experimental animal, its microbic flora, and the test diet may be more critical than realized, the answers to three questions seem of especial importance. First, if animals can maintain a "cariogenic" flora, at what level do they carry it when the experiment is started? Second, if the animals have acquired a "cariogenic" flora, is the right type of diet being used to induce caries? Third, what are the effects of pre-experimental conditions or treatment, and the experimental regimen itself, on the bacterial ecology of the animal?

Lines of hamsters can be raised with either a high or low potential for caries. Present findings now suggest that this depends upon the presence or absence of a penicillin-sensitive flora. Preliminary studies suggest that females transmit this flora to offspring during the suckling period, and other findings indicate that it can be inoculated with faecal material from caries-active animals. In a random sample of hamsters the "cariogenic" flora may not be present in sufficient quantity to induce caries even when a diet with a caries-inducing potential is fed. Although work is now in progress to identify the specific organisms responsible for activity, recent studies of the relationship of the alimentary ecology of hamsters to their caries activity (ROGOSA, JOHANSEN and DISRAELY, 1957) indicate that this is a complex problem.

FITZGERALD, JORDAN and POOLE (1957) found a significant difference in caries activity in rats when penicillin was added to the caries-test diet for 21 days prior to

the feeding of untreated diet. These workers concluded that the penicillin-sensitive flora was an important factor in the higher level of caries activity which follows the early exposure of rats to an experimental ration. In the presently reported study with Osborne-Mendel rats, caries activity was lower in those animals whose penicillin-sensitive flora had been depressed during the suckling period. When these treated animals were exposed to caries-active rats, activity reverted back to usual levels in all areas except along smooth surfaces. These findings in hamsters and rats demonstrate that the flora the animals possess and acquire has an important effect on the subsequent pattern of caries and its level of activity. This finding also indicates that an indigenous "cariogenic" flora can be transmitted between members of the same strain, i.e. from an animal that carries it to one that does not. BELDING and BELDING (1943) felt that caries had been induced in a few rats after the inoculation and transmission of a human strain of streptococci; but only after the strain had been adapted to rats by subcutaneous injection into these animals.

It now appears that, while a "cariogenic" flora can be transmitted between members of the same strain and from female to offspring, it is not so ubiquitous in the general environment as previously supposed and may require considerable time to establish itself at a pathogenic level in the animal. Work now in progress suggests that there may be limitations either to the extent the flora of one species can be transmitted to another or to the degree it will be pathogenic if it is transmitted. This may explain, in part, why previous attempts to induce caries in laboratory animals by the inoculation of non-indigenous strains of bacteria have been generally unsuccessful (MCINTOSH, JAMES and LAZARUS-BARLOW, 1924; MELLANBY, 1930; ROSEBURY and KARSHAN, 1931; LILLY, 1932; BELDING and BELDING, 1943; PALMER, LAFFER and FABER, 1955). In this regard the work of ROGOSA, DISRAELY and JOHANSEN (1958, 1960) and that of JORDAN, FITZGERALD and FABER (1959) indicates that there are considerable differences between the nutritional requirements of strains of lactobacilli isolated from hamsters and rats. "Even when the cultural and biochemical characteristics of certain species were identical with those of isolates from other sources, their nutritional behaviour was not the same" was stated by ROGOSA, DISRAELY and JOHANSEN (1960), and these workers also concluded that each species supports its own characteristic flora.

Along these lines, it has been interesting to find that when Osborne-Mendel rats, which have a high caries potential, have been caged with N.I.H. Black rats, which have a low caries potential, it has not been possible to augment activity in the latter "strain" (STEPHAN and HARRIS, 1954; KEYES, unpublished data).

The question raised in regard to caries-test diets cannot be answered definitively at present. On the basis of work with germ-free rats, ORLAND (1955) concluded that "in the complete absence of bacteria, but not of food debris, no caries developed, and in the complete absence of food substrate, but not of bacteria, again no caries occurred". It now appears that unless a flora with a cariogenic potential is present in sufficient quantity and at the right time, caries will not develop either in the presence of bacteria or with a diet having a caries-inducing potential or in teeth covered with debris. Because the "cariogenic" properties of a diet depend upon the caries-inducing

potential of a bacterial flora, the terms "caries-producing" and "cariogenic" imply activity of an associated microbic complex (ORLAND *et al.*, 1955). For this reason the term "caries-test diet" seems preferable.

One type of caries-test diet does not have the same "cariogenic" potential for all species and strains (STEPHAN and HARRIS, 1954, 1955; JOHANSEN and KEYES, 1955; HUNT *et al.*, 1955; KEYES, 1958). If any of the factors discussed above have a significant influence on the initiation and course of experimental caries, it is surprising, in view of the morphological, physiological, biochemical and ecological differences between animals, that caries-test diets are not more limited in their caries-inducing potential than they are.

Caries active and inactive animals have been observed under numerous laboratory conditions and often have been classified as "susceptible" and "resistant". While the active animal is obviously susceptible, it may be incorrect in the general biological sense to classify the inactive animal as "resistant". A number of factors in the animal-flora-diet complex need investigation before the designation of "resistant" can be considered valid. To avoid the possibility of erroneous conclusions, different diets should be tested, and it should be determined whether the animals carry a flora with a "cariogenic" potential. For example, in other laboratories Hunt-Hoppert "resistant" rats (HUNT, HOPPERT and ROSEN, 1955) have developed considerable activity when fed caries-test diets other than the Hoppert-Webber-Canniff diet (HOPPERT, WEBBER and CANNIFF, 1932; STEPHAN and HARRIS, 1954, 1955; KEYES, 1958; SCHWARTZ, RESNICK and SHAW, 1958). Investigations of genetic factors must take into account the possibility that "resistant animals" have become inactive because the flora has been reduced or eliminated by selective breeding, or that a "cariogenic" flora may be present but the diets used may not activate it.

The answer to the third question is also difficult, but it now seems necessary to consider that changes in the bacterial status of the animal can cause variations in caries activity, and this status can apparently be altered by diets and other procedures during the prenatal, suckling or experimental period. These findings suggest that alterations probably induced in the bacterial ecology of females and weanlings may have contributed to the differences in caries activity seen in animals used to assess the relationship between dietary-developmental factors and tooth resistance (SOGNNAES, 1948; SHAW and SOGNAES, 1955; COX *et al.*, 1956; KEYES, 1956; STEINMAN, HARDINGE and WOODS, 1958; and others).

For this reason refinements in experimental methods seem necessary to clarify many facets of the experimental caries problem. Although the methods have yet to be worked out, it is now clear that some type of microbic equalization and bacterial standardization is essential to permit definitive answers and valid interpretations in studies designed to assess the influence of nutritional effects (prenatal, lactation, etc.), genetic factors, dietary variables and systemic conditions on experimental dental caries. It now seems essential to start with animals which have been equalized microbically, e.g. preconditioned by the inoculation of a standard microbic flora, after the variable and uncertain initial flora has been purposely depressed.

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INFECTIOUS AND TRANSMISSIBLE NATURE OF DENTAL CARIES

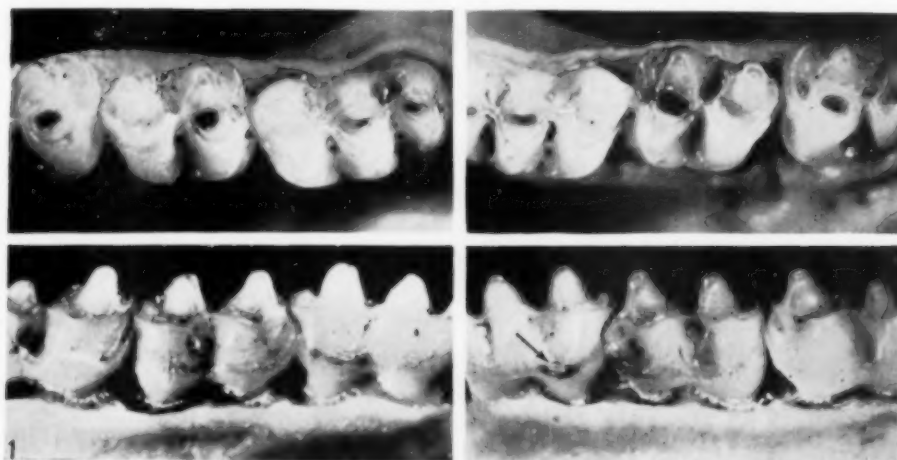


FIG. 1. An example of the negligible caries found in hamsters which do not carry the "cariogenic" flora. In the four quadrants shown, only small areas of enamel pigmentation and one minute lesion (indicated by arrow) are evident.

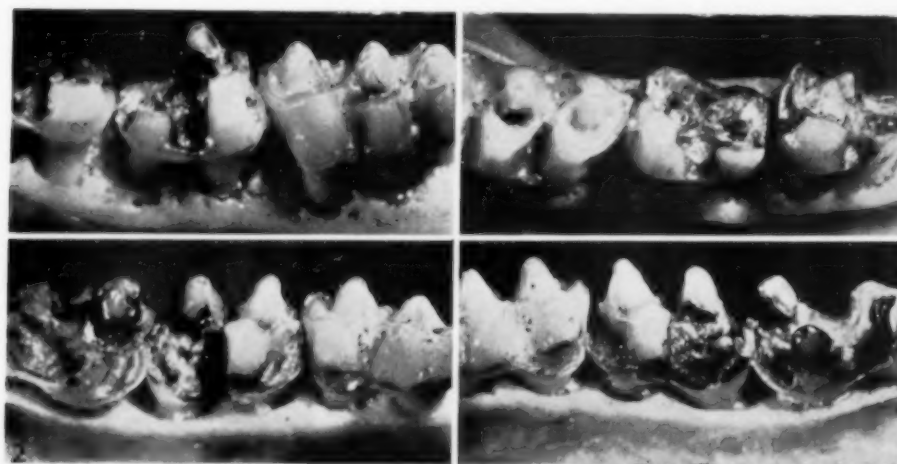


FIG. 2. Caries has involved all twelve molars of a litter-mate to the animal whose jaws are shown in Fig. 1. This typical amount of destruction (scoring 120) can be induced by inoculation of faeces or by exposure to caries-active animals.

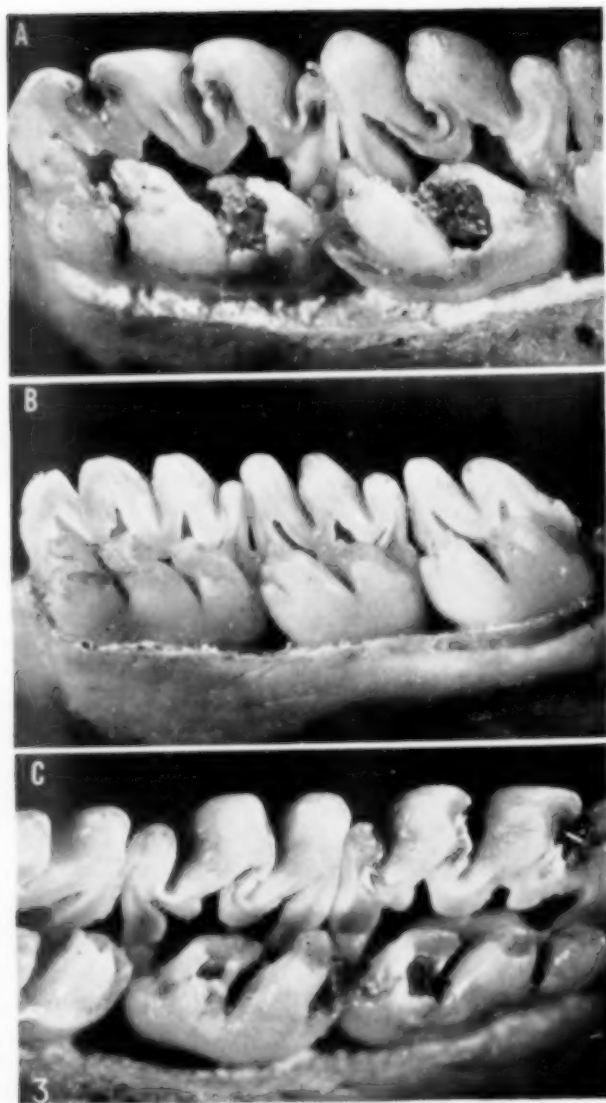


FIG. 3. (A) Jaw from control Osborne-Mendel rat fed test diet for 50 days. (B) Jaw from animal fed penicillin during the suckling period and the test diet for 50 days. (C) Jaw from litter-mate fed penicillin during the suckling period but placed with caries-active animals while on the caries test diet.

THE RESPONSE OF THE ORAL MUCOSA AND PERIODONTIUM TO SIMULTANEOUS ADMINISTRATION OF CORTISONE AND SOMATOTROPIC HORMONE IN YOUNG ADULT MALE RATS

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Abstract—Long-Evans strain, male rats weighing an average of 302 g at the beginning of the experiment, were divided into three groups. Animals in Group 1 received an injection of 5 mg of cortisone three times weekly for a total of 4 weeks. Animals in Group 2 received simultaneous injections of 5 mg of cortisone and 0.5 mg of somatotrophic hormone (STH) three times weekly for a total of 6 weeks. Animals in Group 3 were kept as untreated controls.

It was noted that the cortisone-injected animals died earlier and lost more weight than did the STH-cortisone-treated animals. Histologically, STH appeared capable of compensating for the cortisone-induced reduction in the cellularity of the periodontal membrane and alveolar osteoporosis. Cortisone, on the other hand, appeared to reduce the previously noted oral epithelial response to STH administration. This apparent counteraction by cortisone of the STH effect on oral epithelium was also noted in preliminary tissue-culture studies.

INTRODUCTION

ADMINISTRATION of cortisone has led to an alveolar osteoporosis in some species studied (GLICKMAN, STONE and CHAWLA, 1953; APPLEBAUM and SEELIG, 1955). Alveolar osteoporosis has also been induced in experimental animals by various dietary deficiencies (GOLDMAN, 1954; CHAWLA and GLICKMAN, 1951; FRANDSON, BECKS, NELSON and EVANS, 1953). Somatotrophic hormone (STH) administration to oral tissues of intact rats has resulted in an increased endosteal bone deposition, increased cellularity of the periodontal membrane and an enlargement of the oral tissues at the site of injection (STAHL and JOLY, 1958; STAHL, GERSTNER and JOLY, 1958a; BAUME, BECKS and EVANS, 1954a,b). Oral injections of another protein, crystallized bovine plasma albumin (Armour), did not cause these changes (STAHL, GERSTNER and JOLY, 1958b). STH stimulation has also inhibited osteoporotic changes in alveolar bone associated with some dietary imbalances (BAVETTA, BERNICK and ERSHOFF, 1956; STAHL, GERSTNER and JOLY, 1959).

The present study was undertaken in order to determine whether STH, administered simultaneously with cortisone, may alter the oral tissue response to cortisone described previously.

MATERIAL AND METHODS

Thirty-five Long-Evans strain, male rats weighing an average of 302 g at the beginning of the experiment were divided into three groups.

Group 1. Ten animals received an injection of 5 mg of cortisone three times weekly into the buccal mucosa opposite the maxillary left first molar. Five animals received similar injections into the hind leg.

Group 2. Ten animals received injections of 0.5 mg of STH three times weekly into the buccal mucosa opposite the maxillary left first molar and simultaneously 5 mg of cortisone into the hind leg. The STH was injected in 0.1 ml of distilled water buffered to a pH of about 10.0. Five animals received simultaneous injections of 0.5 mg of STH and 5 mg of cortisone three times weekly into the buccal mucosa.

Group 3. Five animals were kept as untreated controls.

At the end of the experimental period or at expiration, the animals were decapitated and specimens of buccal mucosa and the maxilla were dissected out. The specimens were placed in formalin and prepared for histologic examination. Some buccal mucosa samples from the STH-cortisone-treated animals were grown in tissue culture for cytological examination of the tissues migrating from the explants. These cultures were maintained in Carrel flasks for 2 weeks, without further hormonal treatment, by methods previously described (STAHL, GERSTNER and JOLY, 1958a).

GROSS OBSERVATIONS

Animals receiving cortisone alone lost an average of 84 ± 2.4 g during 4 weeks of experimental procedure and only two animals survived longer than 4 weeks. Animals receiving both STH and cortisone lost an average of 67 ± 14.3 g during 6 weeks of experimental procedure and eleven animals survived 6 weeks of the experiment.

HISTOLOGICAL OBSERVATIONS

Cortisone treated animals

Cortisone-treated animals showed marked inflammation at the injection site especially noted in the orally injected animals. The oral epithelium at the site of injection appeared hyperkeratotic. The underlying connective tissue showed a marked inflammatory infiltrate with associated connective tissue degeneration (Figs. 1 and 2). Two animals exhibited destruction of the entire alveolar septum in association with the local inflammation. It was of interest that the epithelial attachment level in these animals was located at the cemento-enamel junction even though the entire interdental septum had been resorbed (Fig. 3).

The interradicular septum of the maxillary first molar was used as the examination site for changes in the alveolar bone and periodontal membrane. The alveolar bone showed enlarged marrow spaces. A reduction in the cellularity of the periodontal membrane was also noted. The epithelial attachment level was not affected (Fig. 4).

STH and cortisone-treated animals

The oral mucosa showed an inflammatory infiltrate. The epithelium appeared acanthotic. Binucleate cells, which previously have been observed frequently in the oral mucosa of STH-treated, orally injected animals (STAHL, GERSTNER and JOLY, 1958b), were noted with much less frequency in these animals (Figs. 5 and 6).

In tissue culture both fibroblasts and epithelial cells migrated from the STH-cortisone-treated tissues in Carrel flasks at about 3 days after explantation. At 7-8 days, fibroblastic outgrowth was about two-thirds of the fibroblastic migration seen in untreated controls, while epithelial outgrowth approximated one-half to two-thirds of that in the controls. Cytologically neither fibroblasts nor epithelium appeared significantly different from the normal control cells.

The alveolar bone did not show the enlarged marrow spaces noted in the cortisone-treated animals and the periodontal membrane appeared more cellular regardless of the site of hormone injections. The epithelial attachment level was at the cemento-enamel junction (Fig. 7).

COMMENT

Many investigators have demonstrated the ability of STH to counteract disorders aggravated by glucocorticoids (ASTWOOD, 1955; SELYE, 1954; TAUBENHAUS, 1953). The present study, emphasizing the specific periodontal response, also revealed a compensatory or sparing action by STH which appears capable of reducing the cortisone-induced alveolar osteoporosis and reduction in cellularity of the periodontal membrane. These observations extend recent findings of a compensation of cortisone-induced osteoporosis by oestrogen administration (GLICKMAN and SHKLAR, 1954, 1955), to another hormone, STH, which also appears capable of exerting a similar compensatory action.

Of further interest is the apparent ability of cortisone to reduce the response of oral epithelium to STH stimulation which has been described in a previous study (STAHL, GERSTNER and JOLY, 1958a). These histologic findings are similar to preliminary tissue-culture observations which indicate an apparent neutralization of some individual effects of STH and cortisone on oral epithelium and connective tissue when the two hormones are administered simultaneously *in vivo*. The premature and foetal-like epithelial outgrowth of oral mucosa seen in tissue cultures after STH treatment *in vivo* appears to be inhibited, and the mucosa itself less altered cytologically when cortisone is combined with the STH. The significance of these observations needs further investigation.

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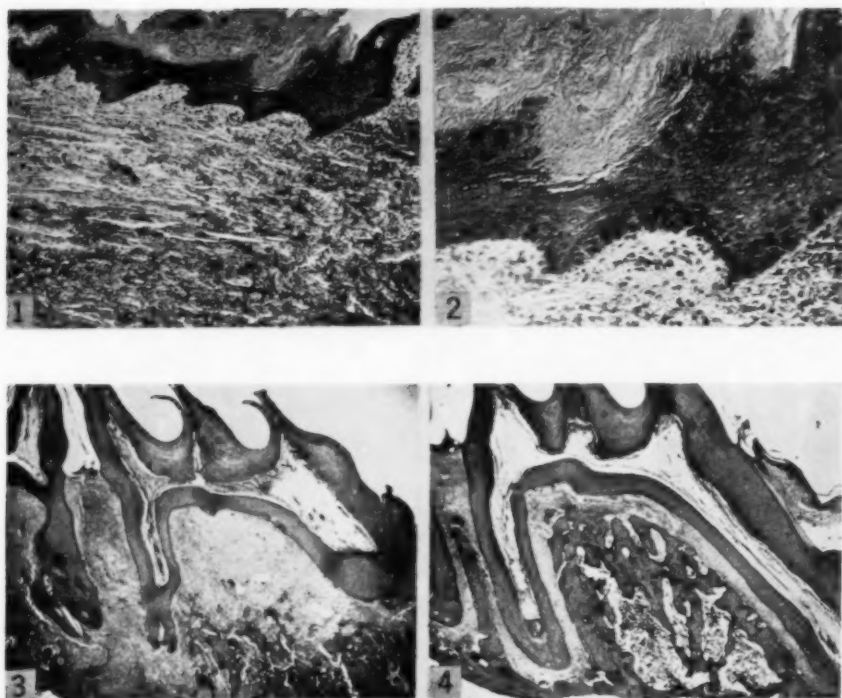


FIG. 1. Section of buccal mucosa (injection site) from an animal receiving 5 mg of cortisone three times weekly over a period of 5 weeks, showing hyperkeratosis and marked inflammation. Haematoxylin and eosin. $\times 45$.

FIG. 2. A higher magnification of the section shown in Fig. 1. $\times 132$.

FIG. 3. Mesio-distal section of the maxillary left first molar from an animal receiving oral injections of 5 mg of cortisone three times weekly over a period of 4 weeks. Note the resorption of the interdental and interradicular septum. Haematoxylin and eosin. $\times 15$.

FIG. 4. Mesio-distal section of the maxillary left first molar from an animal receiving injections of 5 mg of cortisone into the hind leg three times weekly for a period of 4 weeks. Note the enlarged marrow spaces. Haematoxylin and eosin. $\times 15$.

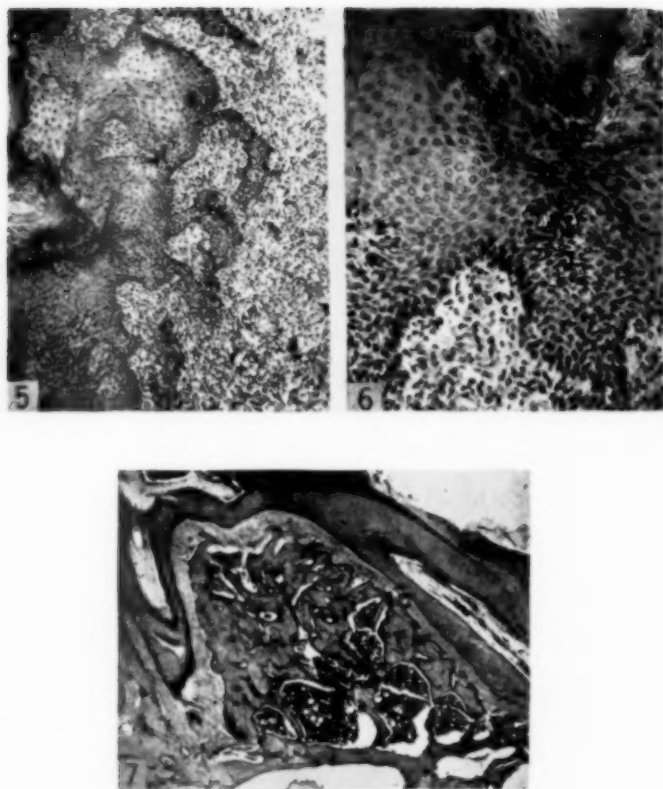


FIG. 5. Section of buccal mucosa (injection site) from an animal receiving simultaneous injections of 5 mg of cortisone and 0.5 mg of STH three times weekly over a period of 6 weeks. Note the epithelial overgrowth and the marked inflammatory infiltrate. Haematoxylin and eosin. $\times 45$.

FIG. 6. A higher magnification of the section shown in Fig. 5. Note the lack of binucleated cells in this area of epithelial growth. $\times 132$.

FIG. 7. Mesio-distal section of the maxillary left first molar from an animal receiving simultaneous injections of 5 mg of cortisone and 0.5 mg of STH three times weekly over a period of 6 weeks. Note the reduction in size of the marrow cavity as compared with Fig. 4. Haematoxylin and eosin. $\times 15$.

RESPONSE OF EMBRYONAL ODONTOGENIC EPITHELIUM IN THE LOWER INCISOR OF THE MOUSE TO 3-METHYLCHOLANTHRENE

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Abstract—The odontogenic epithelium of the Swiss-strain mice has been shown to be extremely susceptible to 3-methylcholanthrene. In our second group of animals, malignant tumours of the odontogenic epithelium were produced in over 80 per cent of the animals in approximately 2-2½ months. The tumours were epithelial, usually squamous in character; however, several showed a variation to a spindle cell or papillary configuration. The tumours destroyed the normal architecture of the mandible and invaded contiguous tissues and in several instances caused death of the animal.

The establishment of the odontogenic epithelium as an easily accessible and highly susceptible tissue to 3-methylcholanthrene allows for further investigation into the physical and chemical nature of the neoplastic cell.

INTRODUCTION

THE frequency with which neoplastic lesions occur in the oral regions places a responsibility on the oral pathologist to participate in the basic study and investigation of the aetiology and nature of neoplasia. In discharge of this responsibility the Oral Pathology Division, Armed Forces Institute of Pathology, has instituted, as part of its research programme, experimental studies into the growth, spread, control and prevention of neoplastic lesions in the oral regions. Several investigators have reported in lower animals, changes in oral tissues ranging from minor alterations to neoplastic lesions, some of them experimentally produced with carcinogenic agents (KOLAS, 1955; LEVY, 1948; LEVY, GORLIN and GOTTSEGEN, 1950; SALLEY, 1954). In other reports, neoplasms of spontaneous origin in the oral regions have been described. Two such articles (GORLIN, CLARK and CHAUDHRY, 1958; SCHLUMBERGER, 1953) describe a variety of oral lesions and tumours in animals.

The results reported on the effects of carcinogenic agents on oral mucous membrane and odontogenic tissues have been variable. LEVY (1948) published the only article we have been able to find concerning the application of carcinogenic substances to the odontogenic tissues in animals. He implanted 20-methylcholanthrene at the apex of the lower incisor tooth in mice and after 16 weeks produced two general types of reaction. One was characterized by hyperplasia of stratified squamous epithelium and the other by formation of a mass of dentine, enamel and cementum growing together in disorderly arrangement.

FLEMING (1952) reported on the early influence of methylcholanthrene on transplanted tooth germs, and his histologic findings resembled the changes noted in our 30-, 60- and 90-day animals. ZEGARELLI (1944) reported 103 spontaneous odontogenic

tumours occurring in seventy-nine Slye stock mice. These lesions were similar in many respects to those produced in our animals. Spontaneously occurring odontogenic tumours of the mixed type have been reported; however, it was questionable whether the lesions were neoplastic.

Benign tumours of the dental apparatus are relatively infrequent (AISENBERG, 1953; BERNIER, 1955; IVY and CHURCHILL, 1930; ROBINSON, 1937; THOMA, 1954); moreover, malignant counterparts are extremely rare (SMALL and WALDRON, 1955). We were interested to know whether the developing tooth under the influence of carcinogenic agents was capable of producing neoplastic growth and whether malignant behaviour of these growths could be induced, and if so, to what extent. The purpose of this experiment was to establish a technique for the production of the neoplastic process in the odontogenic tissues. If such a technique could be established, it would serve as a basis for further experiments planned to study various facets concerning growth, spread, control and prevention of tumours.

METHODS AND MATERIALS

In our investigation two problems presented themselves:

(1) To determine which, if any, odontogenic tissue would give a consistent and predictable response to a specific carcinogen.

(2) To find a vehicle which would allow for efficient and safe deposit of the carcinogenic agent in sufficient quantity in a manner that would permit sustained contact with the tissue over a period of time necessary to produce the desired results.

The first of these two problems was met by selecting the lower incisor of the mouse. The odontogenic tissues at the apical end of the mouse incisor are in a perpetual state of embryonal histomorphologic differentiation. These tissues permit relatively easy access and represented the type of tissue from which it is believed odontogenic tumours arise in the human.

The carcinogen used, 3-methylcholanthrene, is difficult to handle due to its light and powdery nature. This was overcome by using the following vehicle (Avallone Mixture No. 2).

Reagents:

Polyethylene glycol 1500 W;	2.8 g
Polyethylene glycol 4000 W;	1.2 g
3-Methylcholanthrene	1.0 g

3-Methylcholanthrene is a potent carcinogen; therefore, extreme care was necessary. The operator was protected against contamination by using rubber gloves, a surgical face mask and a face shield, a long plastic apron and a long laboratory coat.

For these two respective problems, two groups of female random-bred general-purpose Bagg Swiss-strain mice were used, as follows:

Group 1. Seventeen mice, aged 5 weeks, were subjected to the following procedure: Under Nembutal anaesthesia a linear incision was made along the inferior

border of the mandible. Upon exposure of the bone a hole was drilled with a No. 1 round dental bur in a posterior direction to an area which coincided with the odontogenic soft tissues at the apical end of the incisor (Fig. 1). Into this area a small pellet of carcinogen weighing approximately 4 mg was placed. The tissues were approximated and sutured with 0000 black silk.

These animals were subjected to the following autopsy schedule:

- 2 mice—30 days postoperatively
- 2 mice—60 days postoperatively
- 2 mice—90 days postoperatively
- 2 mice—120 days postoperatively
- 2 mice—150 days postoperatively

Group 2. Twenty-two mice of the same type, 5 weeks of age, were used in the second part of this experiment and were subjected to the same experimental procedures as were the animals in Group 1. Experience gained from the surgical procedures in the first group of animals allowed for more definitive placement of the carcinogenic agent.

RESULTS

Clinical Findings

Group 1

In none of these animals was there clinical evidence of tumour formation. It was decided to observe the remaining seven animals periodically and to perform no more autopsies until tumour formation was evidenced clinically. At 187 days one animal was moribund and showed extensive clinical evidence of tumour. This animal was

TABLE 1. GROUP 1: 17 FEMALE SWISS-STRAIN MICE 5 WEEKS OF AGE AT BEGINNING OF EXPERIMENT

Postoperative days at autopsy	Number sacrificed	Clinical evidence of tumour	Died from tumour	Age at autopsy (days)
30	2	No	—	65
60	2	No	—	95
90	2	No	—	125
120	2	No	—	155
150	2	No	—	185
167-187	—	—	4	—
187	1	Yes	—	222
218	1	Yes	—	253
277	1	Yes	—	312

immediately sacrificed. Four other animals died at night, and though each showed evidence of tumour they were not included in the histologic analysis due to the possibility of postmortem changes. The remaining two animals were placed under daily vigilance. Each showed clinical evidence of tumour formation after 200 days.

They were sacrificed at 218 and 277 days respectively when it was suspected that death from the tumour was imminent (see Table 1). The jaws of the animals were radiographed and prepared for histologic examination.

From the first group of experimental animals we learned that under the conditions described for Group 1, clinical tumour formation could be expected after the fifth month.

Group 2

At 53 days postoperatively, clinical evidence of tumour was observed. On subsequent days several animals showed signs of tumour formation. By 83 days postoperatively, one animal showed tumour to the extent that autopsy was immediately

TABLE 2. GROUP 2: 22 FEMALE SWISS-STRAIN MICE 5 WEEKS OF AGE AT BEGINNING OF EXPERIMENT

Postoperative days at autopsy	Number sacrificed	Clinical evidence of tumour		Histologic evidence of tumour		Age at autopsy (days)
		Yes	No	Yes	No	
83	1	1	—	1	—	118
87	21	14	7	17	4	122

performed. At 87 days postoperatively most of the group showed tumour, some advanced, and it was decided that much would be gained from termination of this group at this time and study of the tumours in their various stages of formation (see Table 2). Several of the advanced tumours were photographed clinically (Fig. 2) and each animal was prepared for histologic examination.

Radiographic Findings

Normal

In the normal adult mouse the embryonic odontogenic epithelium is restricted to the apical third of the inferior surface of the lower incisor. The surgical techniques employed allow for the deposit of the carcinogen in the embryonic epithelium in this area.

Experimental Groups 1 and 2

The radiographic findings in Group 1 at the various postoperative periods up to 150 days are best exemplified in the 30- and 60-day animals.

At 30 days postoperatively the lower incisor showed increased radiopacity and also distortion of the apical third of the tooth. The surgical defect in the incisor erupted to the middle third of the tooth. (The lower incisor of the mouse erupts at the rate of 1-2 mm per week.)

At 60 days, the contour of the apical third of the lower incisor was severely distorted. The middle third of the tooth revealed increased radiopacity and mottling.

The incisal third of the tooth was completely separated from the remainder of the incisor due to the surgical procedure.

Those animals with clinical evidence of tumour in Groups 1 and 2 showed, on radiographic examination, various degrees of distortion and destruction of the mandible and invasion into the surrounding soft tissues.

Histological Findings

Normal

For purposes of comparison, a photomicrograph of a cross-section of a lower incisor of a normal young adult mouse is shown in Fig. 3. All sections shown in the photomicrographs were stained with haematoxylin and eosin. On the superior surface the normal relationship of alveolar bone, periodontal membrane, cementum, dentine and dental pulp, as shown at (A), was seen. The pulp chamber was filled with embryonic connective tissue (B). On the inferior surface the normal relationship of alveolar bone, labial alveolar periosteum, enamel epithelium, enamel, dentine, and dental pulp was seen (C). This state of embryonal histomorphic differentiation may be observed throughout the life of the mouse. The surgical procedures were designed to allow for deposition of the carcinogenic agent in these tissues at (C).

Experimental Group 1

30 days postoperatively. Fragmentation of the root portion of the tooth, with associated fibrosis, dystrophic calcification and chronic inflammation, was noted. The odontogenic epithelium revealed squamous metaplasia and microcyst formation (Fig. 4).

60 days postoperatively. Fragmentation of the tooth and alveolar bone was observed. There was marked fibrosis of the intramedullary tissues, a diffuse chronic inflammation, and multiple cyst formation. The odontogenic epithelium lining the cystic spaces had undergone squamous metaplasia and in some areas proliferated into the surrounding connective tissue (Fig. 5).

90 days postoperatively. The intramedullary tissues showed moderate fibrosis and an infiltrate of chronic inflammatory cells. The cystic spaces were larger than those seen at 60 days, and the metaplastic squamous epithelium revealed changes in size and shape of cells, hyperchromatism and loss of polarity. In several areas the epithelium lining the cysts proliferated into the surrounding tissues.

120 days postoperatively. The tissue revealed a large cystic cavity lined by squamous epithelium. The activity of the epithelium and the inflammatory infiltrate were minimal in comparison to the 30-, 60- and 90-day animals.

150 days postoperatively. The tissue revealed minimal changes. Squamous metaplasia, cyst formation and inflammatory elements were not seen.

187 days postoperatively. An ovoid tumour approximately 1 cm in diameter was examined. The metaplastic squamous epithelium lining the cystic cavity was markedly pleomorphic, hyperchromatic, and showed atypical mitoses and loss of polarity. In some areas the epithelial cells showed a marked morphologic alteration in the form

of spindle-shaped cells (Fig. 6). Invasion of muscle, nerve, glandular elements, bone and periodontal tissues were observed (Figs. 7 and 8).

218 and 277 days postoperatively. In these animals the histologic changes were essentially similar to those at the 187th day.

Experimental Group 2

The 22 animals of this group were sacrificed as follows: one animal 83 days after operation and 21 animals 87 days after operation. Clinical evidence of tumour was observed in 15 of the 22 animals and varied in size grossly from 2 to 15 mm. In these 15 animals, the histologic changes were similar in all respects to those described in the 187-, 218-, and 277-day animals in Group 1. In addition, several of these same 15 animals from Group 2 showed that the metaplastic squamous epithelium lining the cysts had proliferated into the lumen as papillary folds. This epithelium was hyperchromatic and pleomorphic and showed numerous mitotic figures. In several areas keratin pearl formation was noted.

The 7 animals which showed no clinical evidence of tumour in Group 2 were prepared for histologic study. Upon microscopic examination, 3 of the 7 animals showed evidence of tumour. The remaining 4 animals showed histologic changes essentially similar to the 30-, 60- and 90-day animals in Group 1.

DISCUSSION

In Group 1, 17 animals, 10 of which were autopsied at 30-day intervals, showed no clinical evidence of tumour up to 150 days after operation. Of the remaining 7 animals, 5 had developed clinical tumescence at 187 days. The other 2 animals had noticeable tumours at approximately 200 days after the operation. However, in Group 2 (22 animals), 1 had observable tumour at 53 days after operation, and by 87 days 15 animals (approximately 75 per cent) showed clinical evidence of tumour.

The conditions under which these experiments were conducted in relation to uniformity of animals, diet, and carcinogenic agent were standardized. Tumours appeared in Group 2 in less than 60 days, which was much earlier than in Group 1. This variation in time was attributed to improved surgical procedures in placement of the carcinogenic agent in the animals in Group 2.

The studies at 30-day intervals afforded the opportunity to observe histologically the tumour in several stages of its formation. At 30 days after operation, the most significant findings were inflammation, fibrosis, dystrophic calcification, microcyst formation and squamous metaplasia of the odontogenic epithelium. At 60 days the squamous metaplasia and cyst formation were more pronounced, and the inflammatory reaction was markedly fibrotic. At 90 days, the squamous epithelium of the cystic spaces was hyperplastic and showed hyperchromatism, pleomorphism and loss of polarity. Although the epithelium was hyperplastic and showed dyskeratotic changes, evidence of invasion was not convincing. In the 120- and 150-day animals the histologic changes were not as pronounced as those seen in the earlier animals. It is felt that this discrepancy may be explained in one of the following ways: either these animals showed a greater ability for repair, or the placement of the carcinogen

was not as precise as in the earlier animals. The investigators favour the latter explanation.

In the 10 animals observed at 30-day intervals up to 150 days, the histologic changes were similar to those described by ZEGARELLI (1944) as monocystic and multicystic adamantoblastomas, occurring spontaneously in the Slye stock mice. However, we interpreted our lesions to be hyperplastic rather than neoplastic in character.

The animals in Group 1 (187, 218 and 277 days) and the 15 animals in Group 2 which showed clinical evidence of tumour revealed essentially the same histologic changes. The lesions were characterized by cyst formation and squamous metaplasia of the odontogenic epithelium. The squamous epithelium showed pleomorphism, hyperchromatism, loss of polarity and atypical mitoses. The epithelium proliferated in solid masses and strands into the surrounding muscle, gland, nerve, bone and periodontal tissues. In several instances the cyst epithelium proliferated into the lumen, giving a papillary configuration to the tumour. In other instances the epithelium was characterized by a spindle-cell morphology. Serial sections and special stains (Masson trichrome and Wilder's reticulum) convinced the investigators that these spindle cells were epithelial in origin. The only histologic changes noted in the odontogenic mesenchymal tissues were fibrosis, chronic inflammation, and sometimes dystrophic calcification. These changes were interpreted as reaction to the surgical procedures.

The tumours produced in these animals were similar to some of the spontaneously occurring tumours described by ZEGARELLI as the malignant form of adamantoblastoma. Moreover, the tumours in our animals which showed the spindle-cell morphology were considered identical to the sarco-adamantoblastomas described by ZEGARELLI. However, it was our interpretation that these tumours were epithelial rather than mesenchymal in origin.

Acknowledgement—The Avallone Mixture No. 2 was prepared by FRANCIS A. AVALLONE, research assistant, Genito-Urinary Section, Armed Forces Institute of Pathology.

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RESPONSE OF ODONTOGENIC EPITHELIUM TO 3-METHYLCHOLANTHRENE

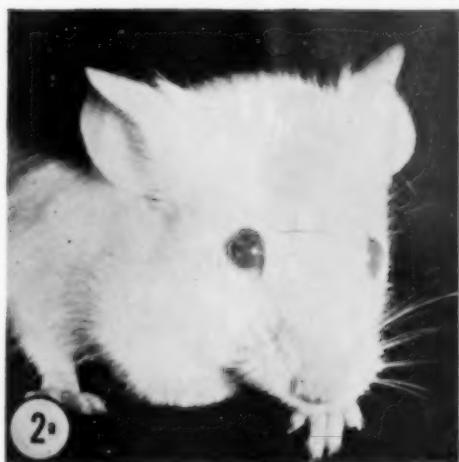
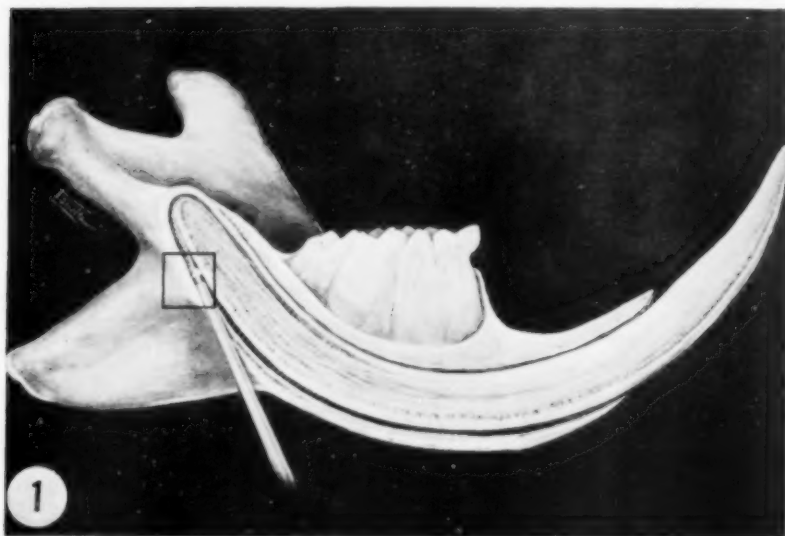


FIG. 1. Carcinogenic agent was placed in odontogenic epithelium in boxed area.

FIG. 2. (a) Frontal view of animal with tumour in the lower right posterior portion of mandible. (b) Ventral view.

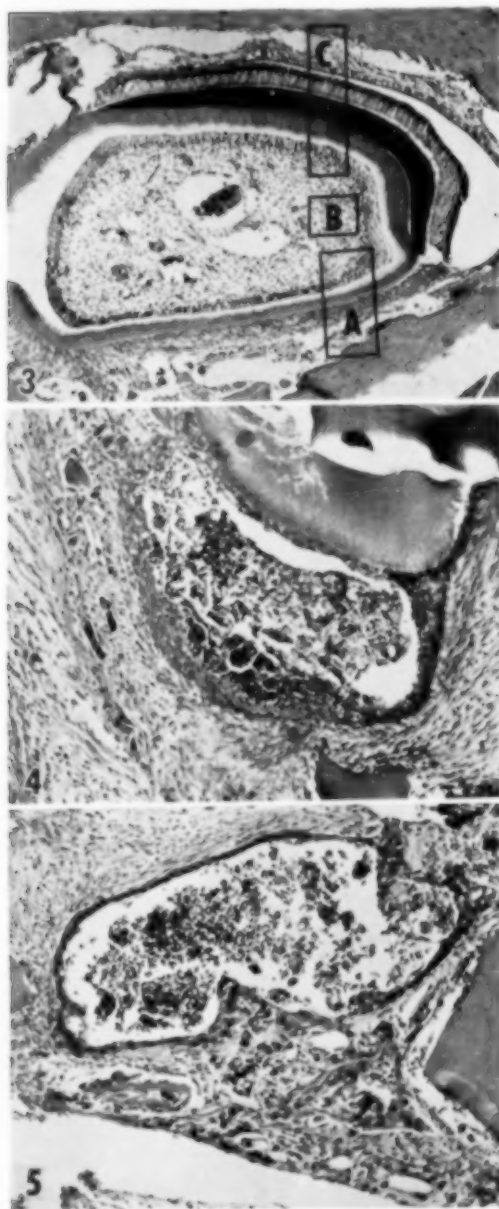


FIG. 3. Cross section, apical third of normal incisor. See text for description of areas (A), (B), and (C). Haematoxylin and eosin. $\times 52$.

FIG. 4. Apical area of root, showing squamous metaplasia and cyst formation, 30 days postoperatively. Haematoxylin and eosin. $\times 88$.

FIG. 5. Cyst lining showing marked activity of epithelium, 60 days postoperatively. Haematoxylin and eosin. $\times 88$.

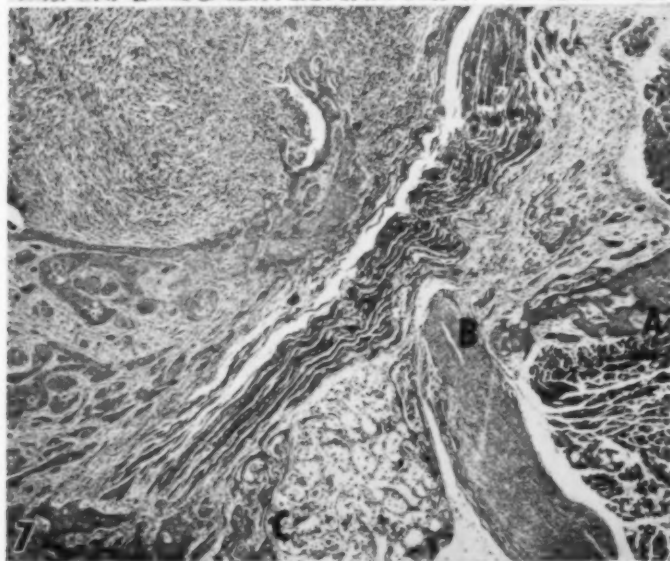
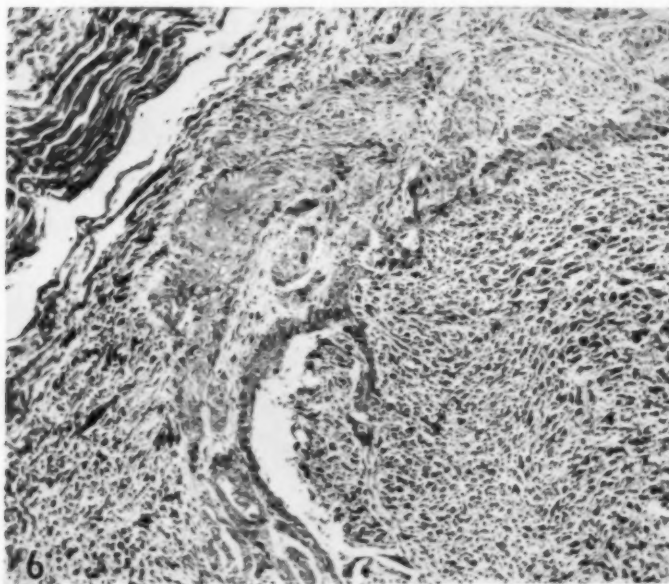


FIG. 6. The tumour formation is characterized by metaplastic squamous epithelium showing loss of polarity and atypical mitoses, as seen in 187-day animal. Haematoxylin and eosin. $\times 88$.

FIG. 7. Section of tumour showing invasion of muscle at (A), of nerve at (B), and of gland at (C). Haematoxylin and eosin. $\times 35$.

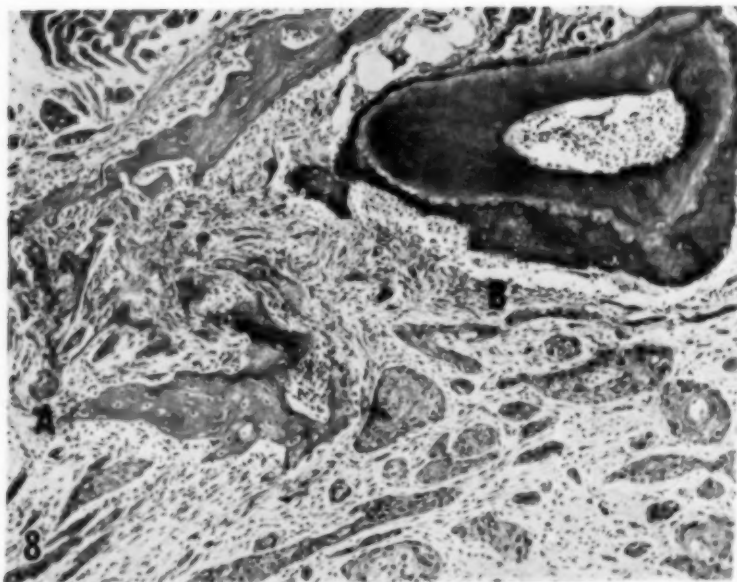


FIG. 8. Section of tumour showing invasion of bone (A), and periodontal tissues (B). Haematoxylin and eosin. $\times 100$.

LYSIS OF RECONSTITUTED COLLAGEN AND CATABOLISM OF PRODUCTS OF COLLAGENOLYSIS BY THE ORAL MICROBIOTA

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Abstract—Broth cultures inoculated with human gingival accumulations catabolized L-hydroxyproline, both as the free amino acid and as combined in L-hydroxyprolyl-glycine or in a digest which was prepared by the action of *Clostridium histolyticum* filtrate on rabbit skin and contained all of its hydroxyproline in peptide form. This fact limits the usefulness of the previously applied determination of liberated hydroxyproline-containing products as a measure of collagenolysis by growing bacterial cultures. A satisfactory quantitative alternative method uses reconstituted collagen as substrate and determines the hydroxyproline content of the undigested residue. A majority of broth cultures of human gingival scrapings lysed reconstituted collagen from rabbit skin at varying rates. A number of strains of human oral veillonellae, fusiform bacilli, streptococci and diphtheroids, singly and in combination, failed to lyse this substrate.

INTRODUCTION

Various members of the oral microbiota attack numerous proteinaceous substrates, including altered collagens from a variety of sources (ROTH and MYERS, 1952, 1956; LUCAS and THONARD, 1955; SCHULTZ-HAUDT and SCHERP, 1955; ROTH, 1957; THONARD and SCHERP, 1957); however, the degradation of native collagens, as in tissue, by these organisms has yet to be demonstrated with certainty. The present experiments concern quantitative determination of bacterial collagenolytic activity by analysis of the undigested residue of a reconstituted collagen. This method is the converse of that used previously, which determined the amounts of hydroxyproline and hydroxyproline-containing peptides released during collagenolysis. This change of method was necessitated by the observation that oral micro-organisms catabolize such products of collagenolysis. The applicability of the previous method is limited accordingly.

MATERIALS AND METHODS

Reconstituted collagen was prepared from the shaved skin of young rabbits weighing from 1.5 to 2.0 kg by a slight modification of the methods of GROSS (1958). The skin was soaked in an aqueous solution containing 5 per cent ammonium chloride and 1 per cent sodium chloride for several days at room temperature, with the addition of several millilitres of chloroform to prevent bacterial growth. Epidermis and subcutaneous fat were then scraped away. In early experiments, the dermis was ground with chips of dry ice in a meat grinder and then dialysed exhaustively against distilled water at 2°C. In later experiments, the dermis was placed under running

tap-water for several hours before grinding. The desalted ground tissue was squeezed as dry as possible through gauze and extracted with 10 volumes (v/w) of 0.5M acetic acid at 2°C for 48 hr. The extract was separated by squeezing through gauze and the residual tissue was extracted with half the original volume of 0.5M acetic acid at 2°C for 48 hr. The combined extracts were clarified by filtration at 2°C through Whatman No. 2 paper coated with Hyflo Super-Cel (Johns Manville); a suitable amount of this filter aid was suspended also in the solution. The filtrates were highly viscous and only slightly turbid and contained from 0.2 to 0.5 per cent collagen, as indicated by their hydroxyproline contents (MARTIN and AXELROD, 1953). Standing for 72 hr at 2°C rendered the filtrates sterile.

For use in collagenolytic tests, an aliquot of such an acetic acid filtrate was transferred aseptically to a Cellophane dialysis sac, previously sterilized by exposure to ethylene oxide vapour, and dialysed against potassium phosphate buffer, pH 7.6, ionic strength 0.4, at 2°C for 48 hr or longer if needed to bring the pH up to 7.0-7.6. Two-ml aliquots of these neutral solutions of collagen were distributed aseptically into sterile culture tubes and incubated at 37°C until a solid gel formed (GROSS, 1958). Eight ml of culture medium (referred to subsequently simply as broth) similar to that used by SCHULTZ-HAUDT and SCHERP (1955) except that acid hydrolysate of casein was substituted for casein, was added to each tube and inoculated with subgingival scrapings or pure bacterial cultures. All test mixtures were incubated at 37°C in an atmosphere containing 5 per cent carbon dioxide and 95 per cent nitrogen (Fig. 1). Bacteria, debris and residual collagen were sedimented by centrifugation at 6000 r.p.m. for 10 min. Both sediments and supernatants were subjected to acid hydrolysis and analysed for hydroxyproline as a measure of residual collagen and specific products of collagenolysis, respectively (MARTIN and AXELROD, 1953; SCHULTZ-HAUDT and SCHERP, 1955). The extent of collagenolysis was calculated from the difference in the residual collagen contents of uninoculated and inoculated tubes. Closely similar procedures were followed in the other tests described subsequently.

RESULTS AND DISCUSSION

Bacterial catabolism of products of collagenolysis. As controls in certain experiments, whole rabbit skin, previously sterilized by exposure to repeated changes of acetone (THONARD and SCHERP, 1957) was exposed to the action of *Clostridium histolyticum* during growth in broth. Although the tissue almost completely disappeared, hydroxyproline-containing substances were barely detectable in either the supernatants or the residues. This result indicated that the micro-organisms had decomposed these products of collagenolysis. This conclusion was verified by such quantitative data as those shown in Table 1. A 0.5 g portion of acetone-dried whole rabbit skin was incubated for 3 days at 37°C in 10 ml of broth inoculated with *C. histolyticum*; a like sample was incubated similarly with 10 ml of a cell-free filtrate of a 48 hr broth culture of *C. histolyticum*. Although the filtrate did not digest the tissue as extensively as did the growing culture, its digest contained 8 times as much hydroxyproline. Again, the culture left very little collagenous residue, as estimated visually and by analysis for hydroxyproline.

TABLE 1. CATABOLISM OF HYDROXYPROLINE-CONTAINING PRODUCTS OF COLLAGENOLYSIS BY *Clostridium histolyticum*

Reaction mixture in 10 ml broth	Hydroxyproline	
	Supernatant ($\mu\text{g/ml}$)	Sediment μg total
0.5 g dry rabbit skin + <i>C. histolyticum</i>	35	50*
0.5 g dry rabbit skin + <i>C. histolyticum</i> filtrate	275	$\geq 400^\dagger$

Incubation period, 3 days.

* No residual tissue visible.

 † Tissue only partially digested.

These results obviously challenged the validity of estimating collagenolysis of tissues by growing bacterial cultures from determinations of the amounts of hydroxyproline-containing products liberated. Accordingly, additional tests were made of the effect of broth cultures of human gingival accumulations and of *C. histolyticum* on L-hydroxyproline, L-hydroxyprolylglycine, and a digest which was prepared by the action of *C. histolyticum* filtrate on rabbit skin and contained all of its hydroxyproline in peptide form (Table 2). The gingival cultures completely consumed the free hydroxyproline and effected a marked reduction in the hydroxyprolylglycine and mixed hydroxyproline-containing peptides of the tissue digest, though not as great as that produced by *C. histolyticum*. It is quite probable that *C. histolyticum* utilizes hydroxyproline by the reaction described by Stickland (reviewed by NISMAN, 1954), in which proteolytic clostridia reduce hydroxyproline to ammonia, carbon dioxide, and unidentified products by coupled oxidation of alanine, valine or leucine.

TABLE 2. CATABOLISM OF HYDROXYPROLINE, HYDROXYPROLYLGLYCINE, AND HYDROXYPROLINE-CONTAINING PRODUCTS OF COLLAGENOLYSIS BY CULTURES OF GINGIVAL ACCUMULATIONS AND BY *Clostridium histolyticum*

Inoculum from subject	Reduction in hydroxyproline* in broth originally containing		
	Hydroxyproline 200 $\mu\text{g/ml}$ (per cent)	Hydroxyprolyl- glycine, 75 $\mu\text{g/ml}$ (per cent)	Digest † , 40 μg hydroxyproline/ml (per cent)
None	0	0	0
A	100	> 74	
B	100	> 74	
C	100	60	37 $\frac{1}{2}$
D	100	20	> 37 $\frac{1}{2}$
<i>C. histolyticum</i>	100	100	

* After anaerobic incubation for 3 days at 37°C.

 † Rabbit skin partially digested by *C. histolyticum* filtrate. \ddagger 100 per cent reduction after 7 days' incubation.

Mediation of such an anaerobic reaction by the common oral bacteria, however, has not been demonstrated.

The failure to demonstrate a release of hydroxyproline and hydroxyproline-containing peptides from periodontal tissue by its indigenous microbiota (THONARD and SCHERP, 1957) might, therefore, mean only that the micro-organisms had degraded these products of collagenolysis. A similar interpretation can be applied to experiments that indicated an inhibition of a clostridial collagenase in growing cultures of gingival micro-organisms (THONARD and SCHERP, 1958). On the other hand, SCHULTZ-HAUDT and SCHERP (1955), THONARD and SCHERP (1957, 1958), and the present authors (unpublished data) have demonstrated regularly the accumulation of hydroxyproline-containing peptides (but not of hydroxyproline) in broth cultures of oral bacteria acting on collagenous tissues sterilized by ethylene oxide. Such altered substrates might be sufficiently more susceptible to proteolysis to liberate these peptides more rapidly than they can be consumed by the bacteria. A satisfactory test of this hypothesis has not been accomplished, owing to technical difficulties. In addition, the hydroxyethyl groups introduced by the treatment with ethylene oxide (FRAENKEL-CONRAT, 1944) might make the collagenolytic products more resistant to bacterial decomposition. This possibility was substantiated by incubating peptides, derived by the action of *C. histolyticum* filtrate on reconstituted collagen respectively untreated and treated with ethylene oxide, with broth cultures of gingival scrapings. Periodically, aliquots were taken from the two cultures and analysed for reduction in total hydroxyproline. The data of typical experiments (Fig. 2) show clearly that hydroxyproline-containing peptides from collagen treated with ethylene oxide are much more slowly catabolized than those from untreated collagen.

Bacterial digestion of reconstituted collagen. An alternative method for determining collagenolysis could measure the decrease of undigested collagen rather than the liberation of hydrolytic products. For our purposes, such a method requires a homogeneous, unaltered collagenous substrate that can be dispensed quantitatively and aseptically; accordingly, it is not readily applicable to intact tissue. On the other

TABLE 3. ACTION OF CRYSTALLINE TRYPSIN ON RECONSTITUTED COLLAGEN FROM RABBIT SKIN AND ON AZOCOLL

Reaction mixture*	Hydroxyproline	
	Supernatant ($\mu\text{g/ml}$)	Sediment (μg total)
Collagen gel + 1 mg trypsin	<12.5	750
Collagen gel + buffer	<12.5	775
50 mg Azocoll + 1 mg trypsin	+	
50 mg Azocoll + buffer	—	

* In 5 ml of phosphate buffer, pH 7.6, ionic strength 0.4; incubated for 16 hr at 37°C.

+, Marked liberation of dye within 3 hr.

—, No liberation of dye after 16 hr.

hand, reconstituted collagen is a relatively pure and readily accessible substrate that meets these requirements. It seems to resemble native collagen fibrils very closely, as shown by electron microscopy (GROSS, 1958) and resistance to trypsin (DRESNER and SCHUBERT, 1955). Our preparation of reconstituted collagen from rabbit skin met these criteria (Fig. 3, Table 3). However, collagen in such fibrils may be more accessible to chemical and enzymatic solubilization than collagen organized in fully mature fibres in tissue (GROSS, 1958).

Representative experiments (Table 4) showed that reconstituted collagen is moderately susceptible to digestion by mixed cultures started with gingival accumulations. In favourable instances, exposure to these cultures for 7 days effected from 20 to 30 per cent reduction in the hydroxyproline content of the collagen samples tested. Longer exposure increased the digestion. These findings indicate that the

TABLE 4. LYSIS OF RECONSTITUTED COLLAGEN BY BROTH CULTURES OF HUMAN GINGIVAL ACCUMULATIONS

Inoculum from subject	Incubation (days)	Reduction in hydroxyproline		
		Expt. 1 (per cent)	Expt. 2 (per cent)	Expt. 3 (per cent)
None	7	0	0	
A	7	30	0	
B	7	23	9	
C	7	15	32	
D	7	0	9	
E	7	15	0	
F	7	23	9	
G	7	0	16	
H	7	8	0	
I	7	23	24	
J	7	23	0	
None	14			0
E	14			43
F	14			19
G	14			0
None	21			0
E	21			60
F	21			23
G	21			19
<i>C. histolyticum</i>	7		>90	

oral microbiota might contribute to the degeneration of collagen fibres seen in periodontitis. The lesser activity of these cultures on collagen, as compared to essentially complete digestion by *C. histolyticum*, is consistent with the chronic nature of periodontal disease, in contrast to the acute character of clostridial myositis.

A number of pure cultures were isolated from representative mixed gingival cultures or from saliva and tested for their action on reconstituted collagen. These

organisms were categorized into the following groups: seven strains of Gram-negative anaerobic cocci (*Veillonella* spp.), six strains of fusiform bacilli, four strains of streptococci, and two strains of diphtheroid bacilli. Singly or in various combinations, these organisms failed to give any indication of collagenolysis after incubation for 7 days. These results support the notion that more than one species of bacteria is involved in the degradation of the collagen substrate employed and support earlier indications of a symbiotic effect of mixed cultures in lysing "collagen paper" (ROTH and MYERS, 1952) or altered collagen (SCHULTZ-HAUDT and SCHERP, 1955). It is quite possible, however, that the oral organisms most active in the degradation of collagenous substrates, as reported here, have yet to be isolated.

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COLLAGENOLYSIS BY THE ORAL MICROBIOTA

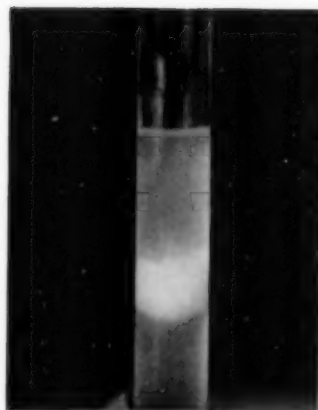


FIG. 1. Typical set up to test for digestion of reconstituted collagen by bacteria. Seven-day anaerobic broth cultures of human gingival scrapings and 2 ml of collagen gel.

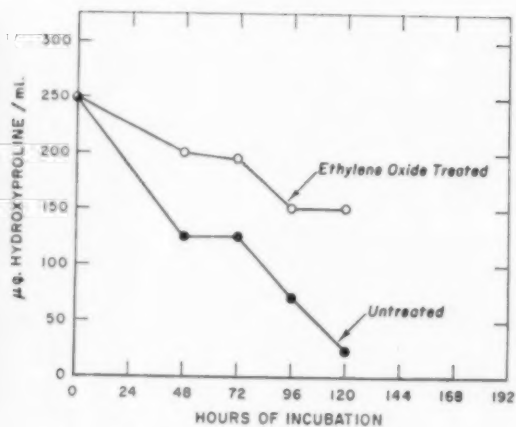


FIG. 2. Comparative catabolism by cultures of gingival accumulations of hydroxyproline-containing peptides in enzymatic digests of untreated and ethylene oxide-treated reconstituted collagen. Anaerobic incubation at 37°C.

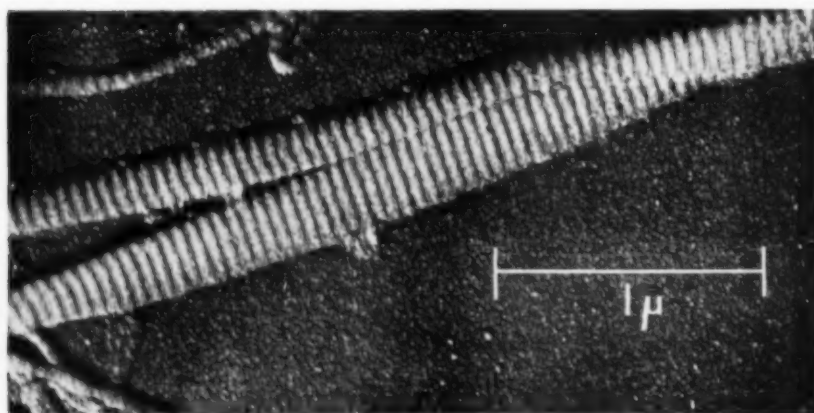


FIG. 3. Electron micrograph of representative fibrils of reconstituted collagen used in present experiments. $\times 32,000$.

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ELECTRON MICROSCOPY OF MATRIX FORMATION AND CALCIFICATION IN RAT ENAMEL

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Abstract—Enamel from incisors and molars of 4-day, 10-day and 1-year old rats has been cut with a diamond knife, as non-decalcified ultra-thin sections, and examined by electron microscopy and diffraction, with the following findings:

(1) Enamel matrix elaboration starts in the ameloblastic cytoplasm, next to the amelo-dentinal junction membrane. Fibrogenesis occurs in two steps: first by an elaboration of diffusely scattered fibrillar protein bundles, embedded in ground substance; secondly, by the appearance of elongated ovoid or frequently cord-like structures, which by coalescence form the enamel matrix. When completed, the fibrillar matrix assumes a three-dimensional reticular appearance.

(2) The apatite crystal growth starts selectively within and on the longitudinal fibrils of the reticulum, in the form of small crystallization centres, which later fuse together, giving rise to strips of calcifying fibrils. Grouping of adjacent calcifying fibrils is followed by partial and then complete fusion, ultimately forming adult apatite crystals, which contain in their core calcified protein fibrils. These crystals take the shape of elongated hexagonal prisms.

(3) In the rat incisor, matrix differentiation and calcification of enamel proceeds progressively from the enamel-dentine junction toward the enamel surface. Three distinct layers can be recognized: inner, middle and outer enamel. The narrow inner and outer enamel contain aggregates of parallel oriented apatite crystals, without any distinct rod structure, whereas the wider middle enamel exhibits a complicated rod interrelationship characterized by alternating layers of rods running in different directions.

(4) In adult rat teeth, a difference in apatite crystallization has been noted both in incisor and molar enamel which is characterized by zones of well-formed apatite crystals intermixed with zones of unfused calcified fibrils.

INTRODUCTION

STUDIES on the matrix-mineral relationship of enamel are of basic importance to a full understanding of calcification in biological systems. With a matrix arising from ectodermal tissue, the enamel ultimately assumes such a high degree of mineralization that its very physical properties pose technical problems to the investigator. It is not surprising, therefore, that very divergent concepts have evolved regarding the organic-inorganic linkage and the enamel rod interrelationships.

The complicated architecture of rodent enamel was first described a century ago by TOMES (1849), but its structure and histogenesis still remain to be adequately studied. The matrix-mineral relationship involves even more minute entities, and until recently has had to be interpreted on the basis of indirect evidence. The difficulty of preserving enamel in decalcified sections has led to one concept which even questioned the very existence of an organic framework in mature enamel, classifying the latter as "acid soluble" (DIAMOND and WEINMANN, 1940).

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If, on the other hand, one accepts the persistence of an organic framework in adult enamel, then the matrix calcification could be viewed, theoretically, as a process whereby the inorganic crystals fill out the spaces between the fibrillar organic framework in such a way that, in the adult enamel, the protein fibrils and the apatite crystals are placed side by side. This view, however, would be incompatible with the low organic content and high specific gravity of calcified enamel. The nature of the inorganic phase has similarly been studied by inconclusive indirect means. HOPPE (1862) and SCHMIDT (1925) proposed the view that, in all stages of developing enamel, the inorganic components are laid down in the matrix in crystalline form. However, VON EBNER (1906) and KITCHIN (1933), using polarizing microscopy, came to the opposite conclusion that the calcium salts in the young enamel matrix are in an amorphous non-crystalline form.

Despite the advantages afforded by newer techniques, these points are not completely understood. Application of electron microscopy to studies of enamel calcification has been restricted to the indirect replica technique, to decalcified specimens or to sections containing only the soft developing enamel matrix. Hence, the nature and relationship between the organic and inorganic ingredients of enamel could not be observed directly through the full sequence of matrix formation and mineralization.

To resolve this problem, it would seem essential to be able to visualize directly under appropriate magnification ultra-thin sections containing both the organic and inorganic elements in their proper relationships without any prior treatment with decalcifying agents. Recently, with the development of the diamond knife, it has become possible to prepare ultra-thin sections of undecalcified teeth at any stage of enamel development (FRANK, 1959).

In this investigation, such sections have been examined by electron microscopy and electron diffraction with the following goals in view: (1) to follow enamel matrix differentiation; (2) to obtain additional information about the inorganic crystallization process; and (3) to study at high magnification the full sequence of steps in enamel rod development in the rat from its initiation to its conclusion.

Only brief mention will be made of the morphological changes in the ameloblast which accompany enamel elaboration, because this study is primarily designed to shed some additional light on the nature of and relationship between the organic and inorganic elements and their distribution within the enamel rods. Elsewhere (FRANK, SOGNAES and KERN, 1960), a comparison is made between human and rat enamel (notably in the more mature stage) from the point of view of the inorganic phase and its relationship to the organic fibrils. In the present study, more extensive observations are recorded regarding early fibrogenesis and the mechanism of rod differentiation in rat enamel where the arrangement is particularly complicated.

MATERIALS AND METHODS

In order to cover the range of cellular differentiation, matrix formation and calcification of the rat teeth, three age groups of albino rats were chosen; namely, 4-day, 10-day and 1-year-old. Both molars and incisors were studied. However, the principal observations were made on the lower incisor teeth because here, in the

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same specimen, comparisons can be made on the full range of development from the earliest matrix formation up to the functional stage of the erupted enamel. Two types of fixation were used: (1) direct fixation in 1% buffered osmic acid immediately after removal of the tooth buds and lasting for a period of 4 hr; and (2) perfusion, in the case of the 10-day old rat tooth germs, of 5 ml of 1% osmic acid through the heart of the animal, followed by post-fixation of the isolated tissue for 3-4 hr in 1% buffered osmic acid. The final blocks measured $\frac{1}{2}$ -1 mm³. Care was taken to orientate the specimens for sectioning with proper reference to tooth position. Tissue orientation was facilitated by the use of special embedding capsules with a fine pointed end. The Servall-Porter-Blum Microtome equipped with a diamond knife was used for the preparation of all sections. Thus, it was possible to prepare the specimens without prior decalcification, irrespective of their degree of calcification. An RCA Model Type EMU-3B electron microscope, which was also equipped for electron diffraction, was used throughout. The electron micrographs illustrating this presentation were prepared partly by direct print from the original plates, and partly as facsimiles of the original plates (i.e. negative prints), as is indicated in the description of each figure.

RESULTS

For the sake of orientation, the diagram presented in Fig. 1 will be referred to repeatedly in describing the several photographic illustrations (Figs. 2-19), after which the depicted sequences, matrix differentiation and apatite crystal growth, are summarized in the last drawing, Fig. 20.

After elaboration of a small layer of dentine, enamel matrix elaboration starts in the distal part of the ameloblast next to the amelo-dentinal junction. The very first enamel matrix to be laid down is a continuous thin sheath of matrix about 0.5-2 μ wide. This layer of pre-enamel matrix, which we shall refer to as the "inner enamel", is continuous over the underlying dentine (Fig. 1A). Soon thereafter, however, strips of pre-enamel matrix differentiate on the lateral side of the ameloblasts within their cytoplasm. These strips of enamel matrix are in continuity with the inner enamel (Figs. 1B and 2) and by further differentiation of matrix, they increase in width and length. As a result of this sequence of events, the ameloblastic cytoplasm appears to extend into the pre-enamel matrix (Figs. 1B, 2, 4, 5, 6).

The distal part of the ameloblast is classically called the "Tomes process" because of its different structural appearance under the optical microscope. From our observations, it would seem more appropriate to consider this portion as a direct continuity of the ameloblastic cytoplasm. The homogeneous appearance of the Tomes process, observed under the light microscope, may be related to the basal migration of the cytoplasmic inclusions (USSING-NYLEN and SCOTT, 1958). As the distal part of the cytoplasm, corresponding to the region described as the Tomes process, becomes devoid of mitochondria and endoplasmic reticulum, it takes on a more transparent homogeneous-looking appearance.

At the stage where pre-enamel matrix has differentiated around the distal part of the ameloblastic cytoplasm (Figs. 2, 4, 5, 6), two distinct types of matrix differentiation have been observed. In both, fibrogenesis takes place in the ameloblastic cytoplasm.

The first type consists of a diffuse appearance of fibrils in the distal part of the ameloblastic cytoplasm. Next to the pre-enamel matrix, intracytoplasmic fibrils can be seen (Fig. 3), and it is possible to follow bundles of such fibrils from the ameloblast to the pre-enamel matrix. We have not been able to distinguish distinct cell membranes between these two structures (Fig. 3).

The fibrous protein elements of the pre-enamel matrix are embedded within the ground substance (Fig. 3). In the present context, the latter term is not used in any specific sense except to denote a non-fibrillar cementing substance of indefinite chemical composition, presumably mucoproteins, which fill the interfibrillar spaces and appear to be more electron dense than the ground substance of the ameloblastic cytoplasm *per se*.

The second type of matrix differentiation is associated with the presence of elongated ovoid or elongated bodies distributed in the distal part of the ameloblastic cytoplasm. These special structures possess a round or oval shape in transverse sections (Figs. 4, 5, 6) and vary considerably in size from 400 Å to more than 1 μ in diameter. It is important to note, however, that these are not actually globular but rather are sections of elongated cords (Fig. 5). Consequently, random sections may appear to contain both elongated and cross-cut globular structures (Fig. 5).

It is noteworthy that these bodies exhibit a varying electron density. At the beginning of matrix differentiation, each is limited by a relatively electron dense membrane which surrounds an amorphous-looking core (Fig. 4). Some fine scattered fibrillar elements differentiate progressively in the cores of these structures, which then become gradually filled out by fibrils embedded in an interfibrillar ground substance (Figs. 4 and 5). Consequently, small cords or strips of pre-enamel matrix are formed throughout the distal part of the ameloblast (Figs. 4, 5, 6). Ultimately these cord-like structures fuse one with the other (Figs. 4 and 6). By coalescence of these elements, a continuous enamel matrix is formed which fills the distal part of the ameloblastic cytoplasm. The stage of development reached by these processes is summarized diagrammatically in Fig. 1C (compared with Fig. 13).

By the time the various zones of enamel matrix formation have fused, a definite orientation can be found in the fibrous protein elements. This is characterized by an almost parallel orientation of long fibrils, which in turn are connected by short transverse fibrils of the same width (Fig. 7). Altogether, this matrix now constitutes a reticulum delineating round meshes filled with ground substance. Fig. 20, No. 1 shows diagrammatically the relationship between the reticulum and the ground substance in the enamel matrix at this stage of development. The width of the fibrils constituting this organic reticulum before the beginning of calcification ranges from about 60 to 160 Å. While inorganic crystal deposition takes place at very early stages of matrix formation in scattered regions of this organic framework, it seems more practical to discuss this question separately.

The sequence of crystallization is illustrated photographically in Figs. 8-11 and diagrammatically in Fig. 20.

Apatite crystal growth starts very soon after differentiation of the fibrous protein and the ground substance. For the sake of orientation, Fig. 8 shows the junction

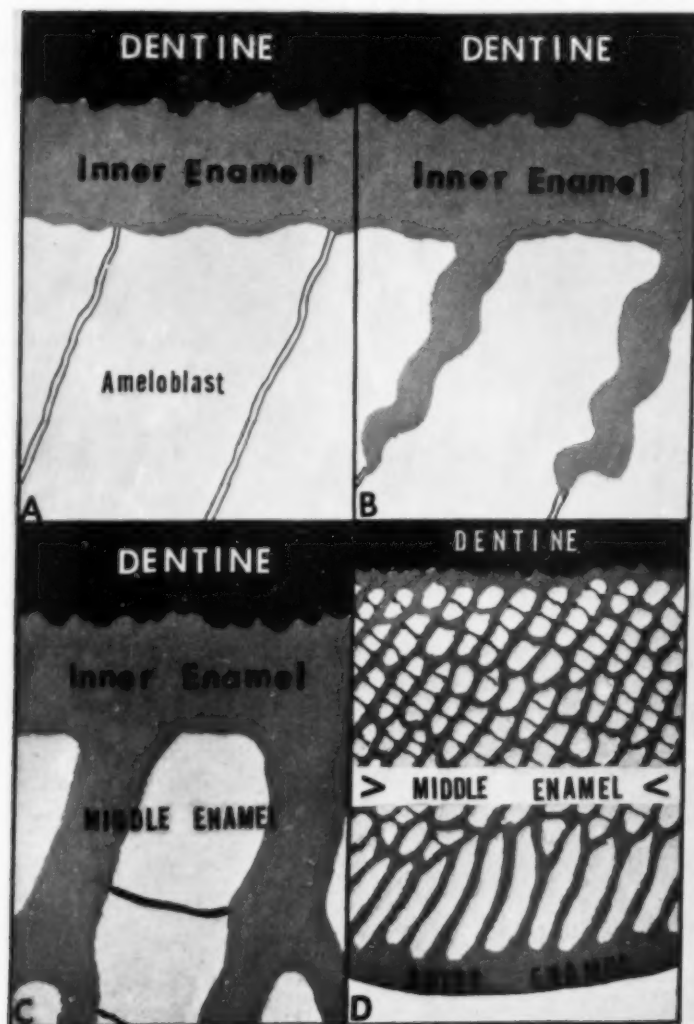


FIG. 1. Diagrammatic representation of amelogenesis in the rat incisor. (A) Formation of inner enamel ($\times 8000$); (B) Beginning of middle enamel development ($\times 8000$); (C) Middle enamel development completed. Note the alternating layers of longitudinally and transversely cut rods ($\times 8000$); (D) Inner enamel, middle enamel (only partially represented) and outer enamel ($\times 1300$).

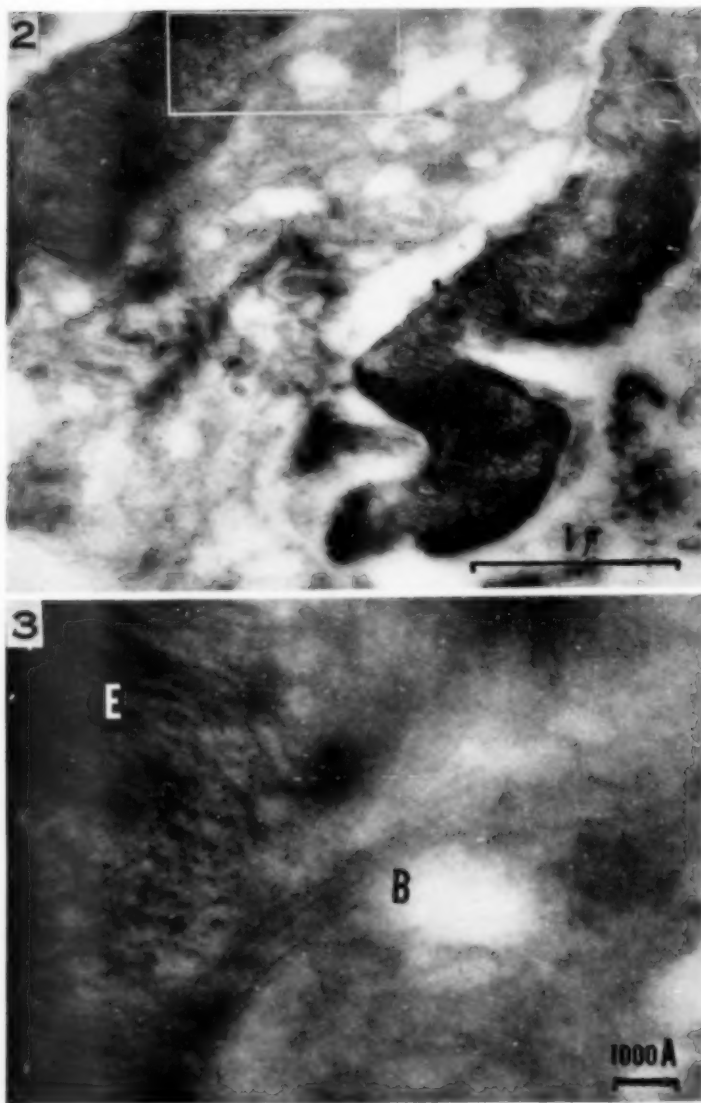


FIG. 2. Non-decalcified section of developing rat incisor showing two strips of pre-enamel matrix (dark) limiting the cytoplasm of one ameloblast. (Direct print $\times 29,000$).

FIG. 3. Enlargement of the rectangular area delineated in Fig. 2. No cell membrane is apparent between the pre-enamel matrix (E) and the cytoplasm of the ameloblast. A bundle of protein fibrils (B) can be followed from the ameloblastic cytoplasm into the pre-enamel matrix. (Direct print $\times 90,000$).

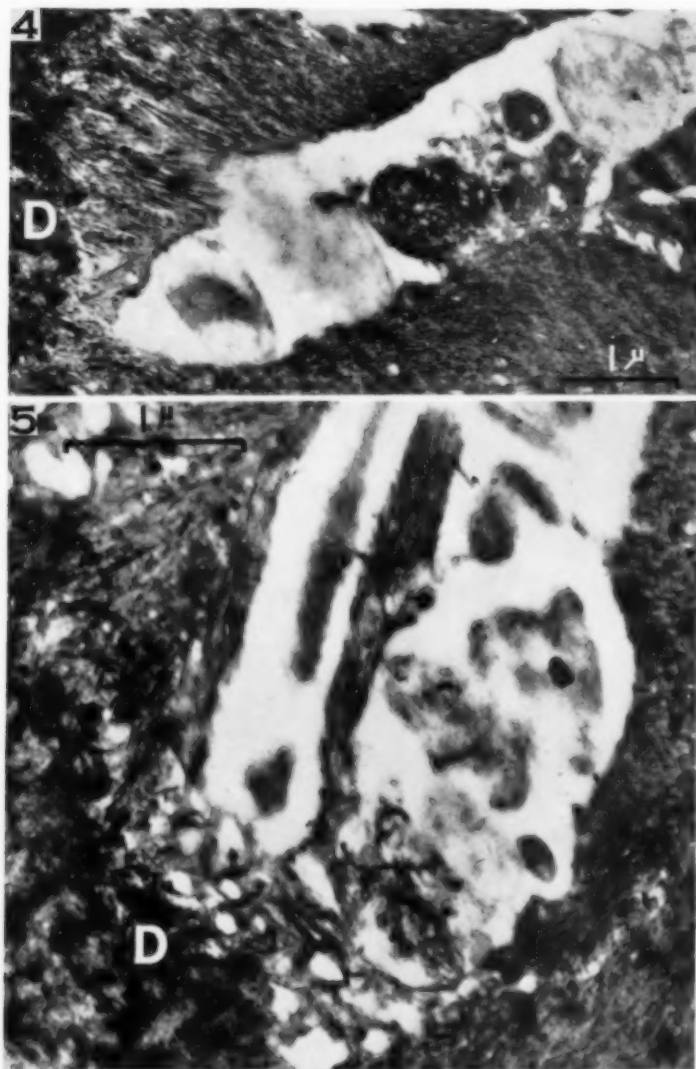


FIG. 4. Non-decalcified section of a developing rat incisor near the enamel-dentine junction. Various stages of enamel matrix differentiation can be seen in the rounded to ovoid bodies contained in the ameloblastic cytoplasm surrounded by enamel matrix already deposited. D=dentine. (Direct print $\times 16,000$).

FIG. 5. Detail of section similar to Fig. 4 shows that the rounded to ovoid bodies containing enamel matrix are actually elongated cords when viewed in longitudinal sections. D=dentine. (Direct print $\times 25,000$).

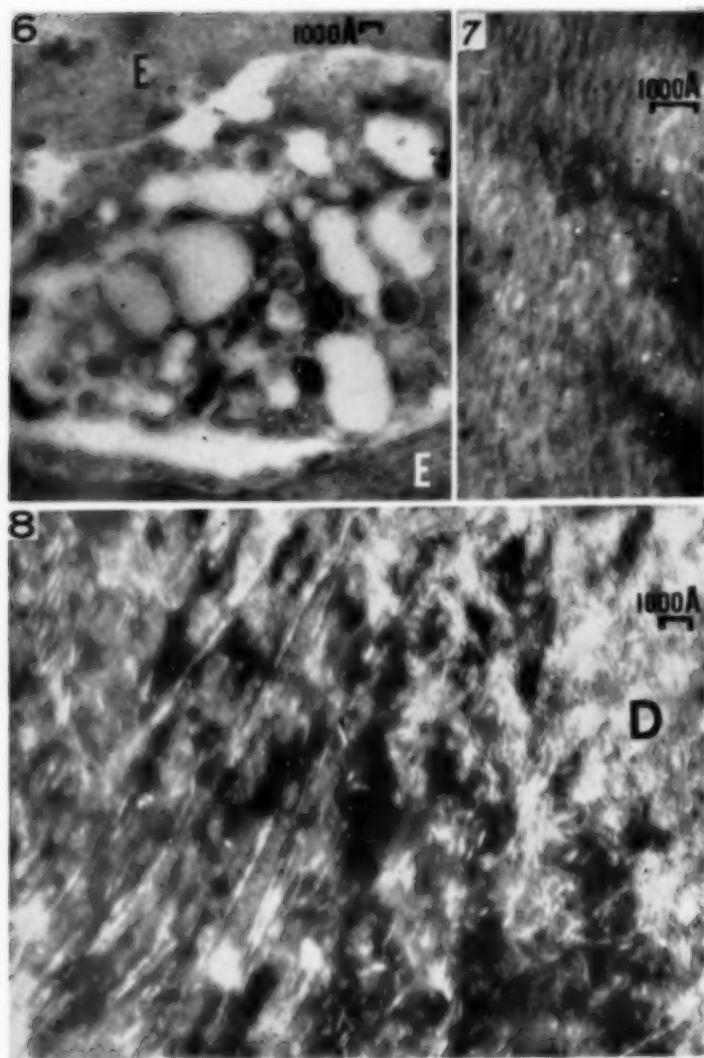


FIG. 6. Differentiation of enamel matrix throughout the ameloblastic cytoplasm surrounded by longitudinal enamel rods (E). Through this sequence the cytoplasmic gap between longitudinally running rods is progressively filled. (Direct print $\times 32,500$).

FIG. 7. Organic reticulum embedded in ground substance, before beginning of calcification as seen in non-decalcified section of a developing rat incisor. (Negative print $\times 65,000$).

FIG. 8. Non-decalcified section of a developing rat incisor along the enamel-dentine junction. Beginning of apatite crystal growth in enamel, appearing as white, electron dense strips. More minute apatite crystals can be also seen in dentine (D). (Negative print $\times 45,000$).

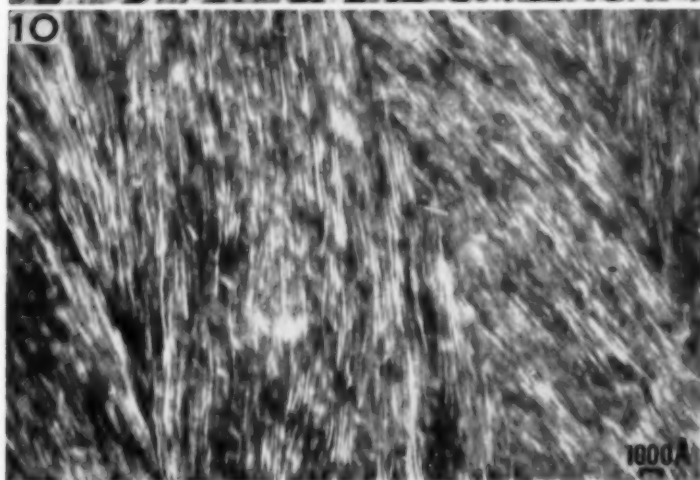
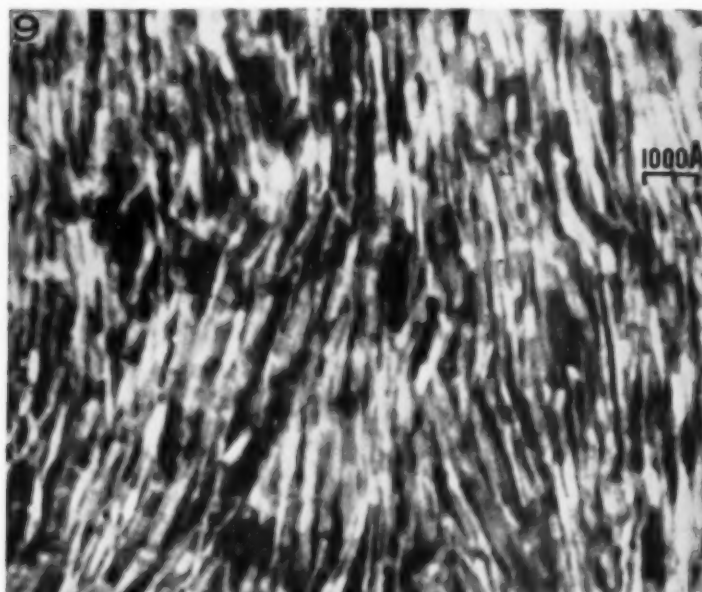


FIG. 9. Part of an enamel rod in the developing rat incisor as seen in non-decalcified section. Different stages of apatite crystal growth can be seen: small crystallization centres appear in the middle upper part as white rounded dots within and on longitudinal protein fibrils. In other areas, white strips of calcifying fibrils as well as partial fusion of adjacent calcifying fibrils are present. (Negative print $\times 78,000$).

FIG. 10. Four enamel rods seen in non-decalcified section of developing rat incisor. Apatite crystal growth is selectively confined to the longitudinal protein fibrils and appears as small electron-dense white strips, which are differently oriented from one rod to another. (Negative print $\times 29,000$).

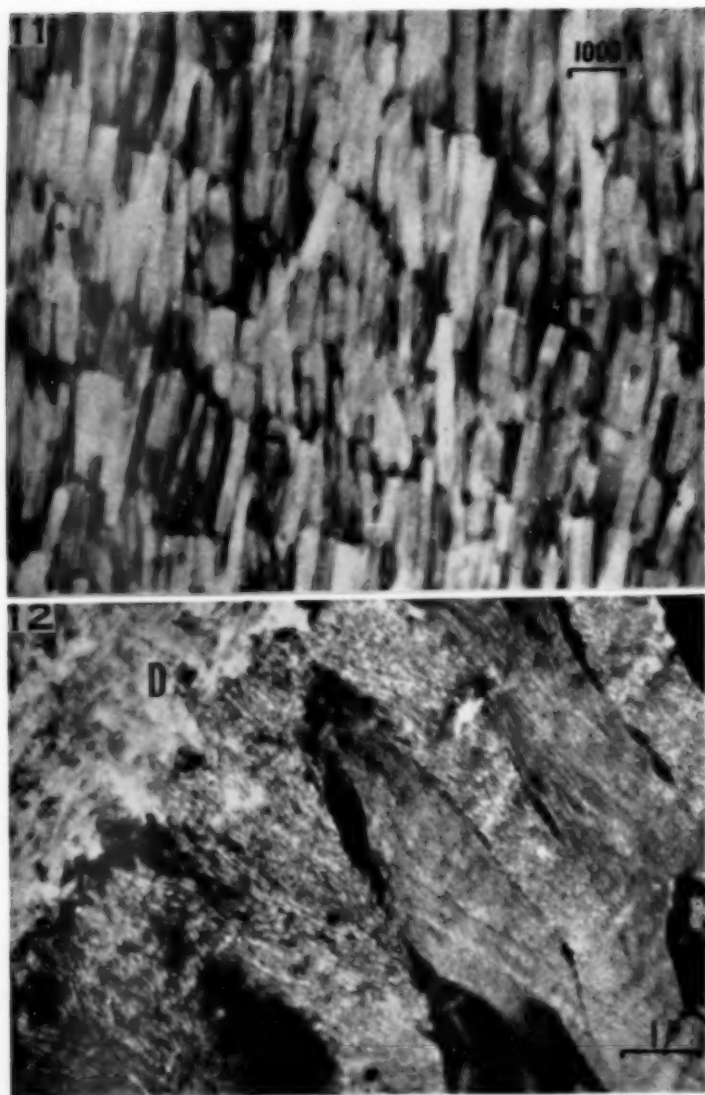


FIG. 11. Longitudinal non-decalcified section of an enamel rod in a 10-day-old rat. The apatite crystal growth is now virtually completed as evidenced by complete fusion of adjacent calcifying fibrils which can be seen by transparency in the core of some apatite crystals. (Negative print $\times 78,000$).

FIG. 12. Developing rat incisor near the enamel-dentine junction showing the filling out of the ameloblast cytoplasmic gaps by enamel matrix. D=dentine. Non-decalcified section. (Negative print $\times 11,500$).

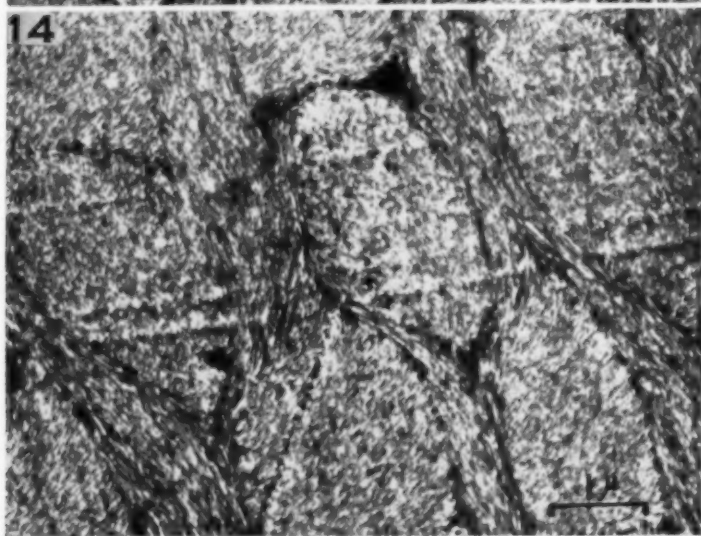
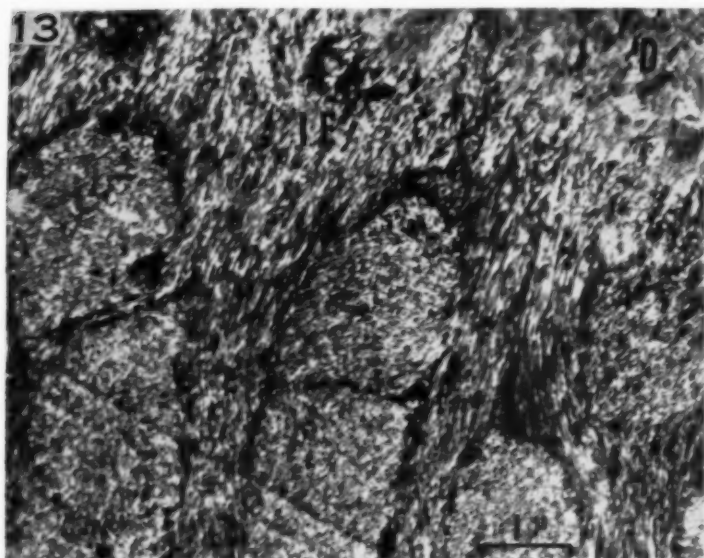


FIG. 13. Longitudinal non-decalcified section of the lower incisor in a 10-day-old rat, showing dentine (D), inner enamel (IE) and middle enamel. In this latter portion, the alternating pattern of the rod direction is clearly visible. (Negative print $\times 13,000$).

FIG. 14. Photomontage corresponding to same area as shown in Fig. 13, deeper in the middle layer of the same enamel. Note the ramification of longitudinally running rods alternating with almost transversely-cut rods. (Negative print $\times 14,000$).

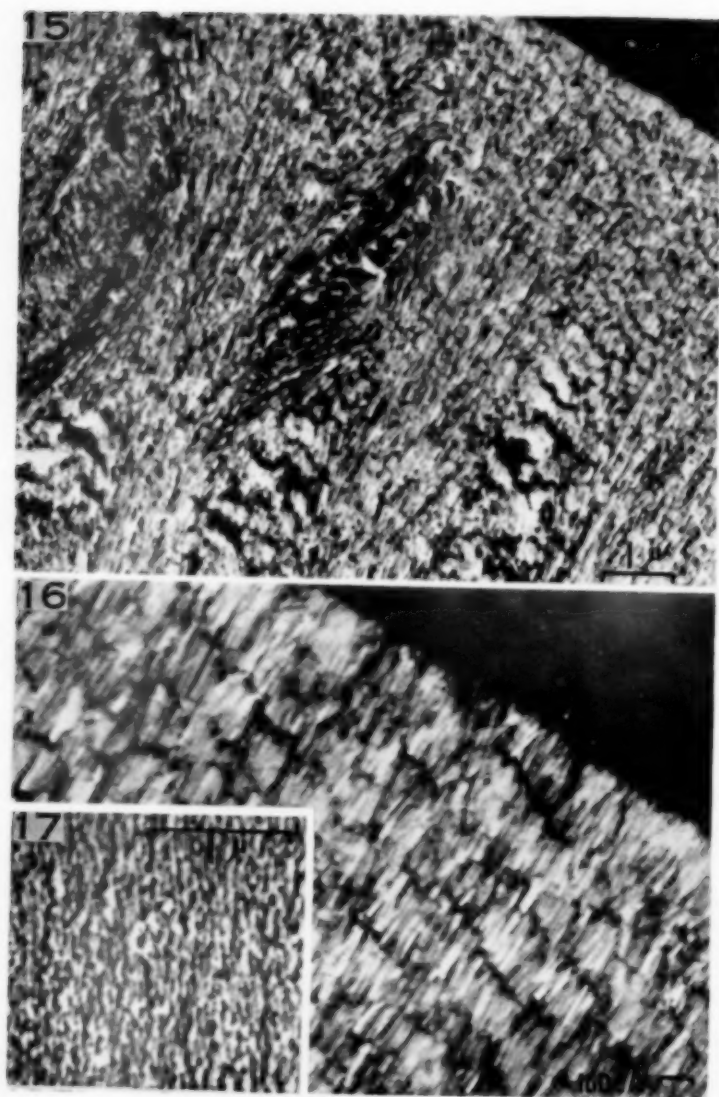


FIG. 15. Non-decalcified section of the lower incisor in 10-day-old rat showing the outer layer as well as the surface of enamel (upper right). (Negative print $\times 10,000$).

FIG. 16. Higher enlargement of Fig. 15 showing apatite crystal aggregates with parallel axes in the outer layer of incisor rat enamel. (Negative print $\times 45,000$).

FIG. 17. Longitudinal non-decalcified section of a 1-year-old rat incisor showing showing a zone of unfused calcified fibrils in an enamel rod. Note the reticular appearance of the calcified protein fibrils. (Negative print $\times 20,000$).

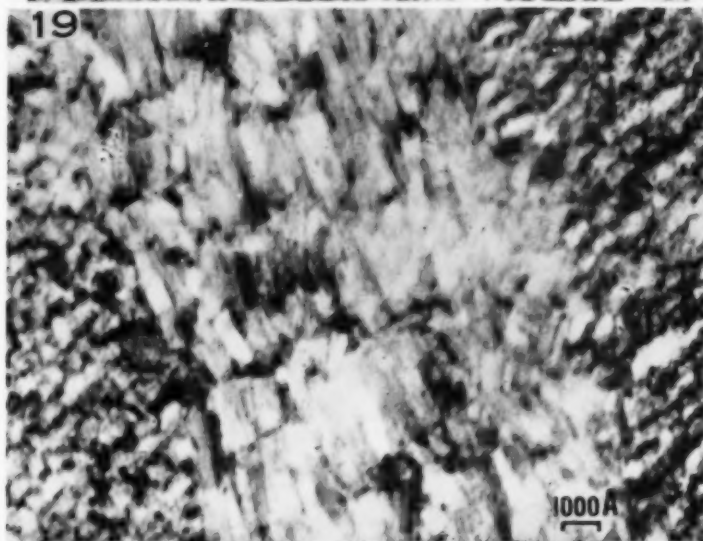
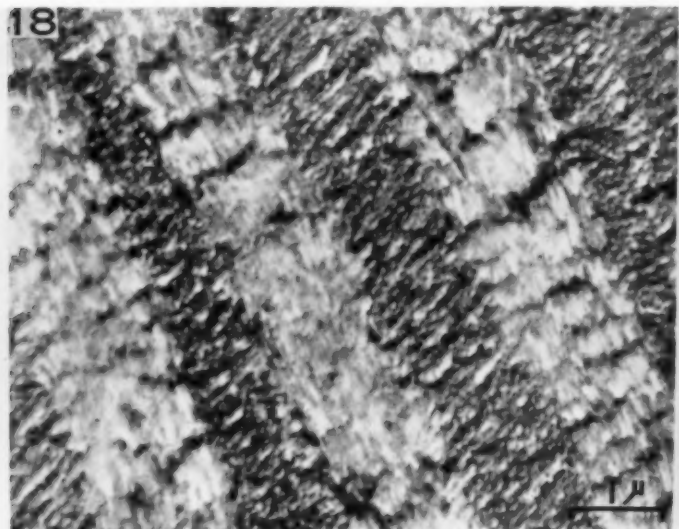


FIG. 18. Longitudinal, non-decalcified section of molar enamel from a 1-year-old rat showing layer of parallel rods. (Negative print $\times 13,000$).

FIG. 19. Higher magnification of a section similar to Fig. 18, showing that in one rod the apatite crystals are cut longitudinally, whereas in the adjacent rods they are cut transversely. (Negative print $\times 52,500$).

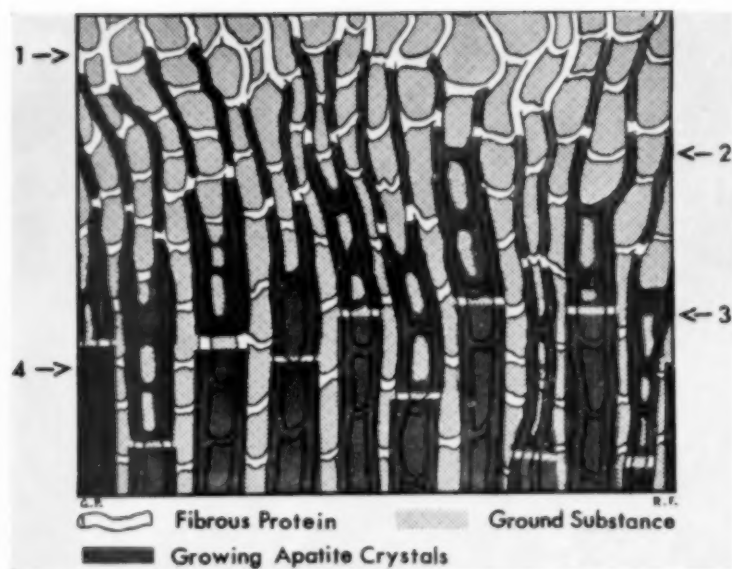


FIG. 20. Diagrammatic representation of the apatite crystal growth in rat enamel. (1) organic matrix formation; (2) beginning of calcification along the longitudinal fibrous protein; (3) partial fusion of adjacent calcifying fibrils; (4) young apatite crystals.

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between dentine and enamel. Where the calcification process starts in the inner enamel layer, it will be noted that there are high-density strips along the fibrous protein. These first crystallization centres, laid down in a preferential orientation, are to be found in scattered regions of the matrix. In other words, the beginning of calcification is selectively confined to the fibrous constituents of the matrix. When viewed at somewhat higher magnification the calcification centres first appear as small electron-opaque dots, deposited exactly on and within some of the longitudinal fibrils previously described (Fig. 9). Small electron-dense lines appear along the longitudinal fibrils by confluence of the initial crystallization centres, which to begin with were separated by less electron-dense areas representing uncalcified parts of the fibrils. This process is in contrast to calcification in dentine wherein a true calcification front exists and wherein calcified dentine is formed along a more or less regular border. In enamel matrix, on the other hand, the calcification proceeds here and there by an irregular distribution of crystallization centres along scattered longitudinally oriented fibres. All the fibrils are not involved at the same time. The crystallization is more advanced in some than in others, as will be noted in Figs. 9 and 10. That these electron-dense areas do in fact represent inorganic crystals was apparent from electron diffraction patterns obtained at this stage, which were typical of the apatite crystal group. The width of the calcified fibrils, ranging from 60 to 200 Å, suggest a slight enlargement of the calcifying fibrous protein in connection with the apatite crystallization. This point can be illustrated by direct observation of the early calcification of individual fibres seen in Fig. 9. Here it will be noted that the electron-dense calcifying strips do extend slightly beyond the width of the uncalcified portion of the fibrils.

Two additional features are apparent at this stage. One is the precise development of the inorganic crystallization along the longitudinal direction of the fibrils as best illustrated in Fig. 10. Furthermore, one can at this stage detect a second phenomenon, namely a partial fusion of individually calcifying fibrils as noted in several areas within Fig. 9. Even within this one single field, one can note three different situations: (1) isolated crystallization centres, spotted like beads on a string, along the fibrils; (2) completely calcified individual protein fibrils; and (3) partial fusion of calcifying fibrils which results in a grouping and fusing of adjacent fibrils (Fig. 9). As the inorganic crystallization process advances, the short transverse fibrils of the reticulum and the spaces filled by ground substance between them become involved, as diagrammatically illustrated in Fig. 20, No. 3. As a result, the future profiles of the adult apatite crystal begin to be outlined in a manner suggestive of the development of a photograph. Little by little, the non-calcified sub-microscopic spaces between calcifying fibrils are reduced. By the time the fusion of adjacent calcifying fibrils is completed, the apatite crystals appear in longitudinal sections as elongated electron-opaque rodlets (illustrated in Fig. 11 and schematically represented in Fig. 20, No. 4). In thin apatite crystals, the fibrous protein elements, now embedded in the core of the crystal, can be seen by transparency (Fig. 11). The largest young apatite crystals having assumed this stage and formed by fusion of the calcifying fibrous protein have now an average width of from 400 to 500 Å.

It must be emphasized, at this stage, that some calcified fibrils can remain unfused, as it will be shown later to be the case in adult enamel. Up to this point, we have primarily considered apatite crystal growth, which proceeds in a scattered fashion in different portions of the enamel matrix. It is now necessary to pursue the further development of the enamel beyond the early inner enamel layer.

According to our observations, the rat enamel is built up by a juxtaposition of enamel rods which can be distinguished from each other by the fact that the long axes of the crystals, while parallel within each rod, have a different orientation within adjacent rods. In other words, adjacent rods do not follow an identical course. Furthermore, the rods do not appear to be separated by any distinct inter-rod regions or rod sheaths comparable to those observed in similar preparations of human enamel and described elsewhere (FRANK, 1959; FRANK, SOGNAES and KERN, 1960).

When the inner enamel is deposited as a thin layer along the enamel-dentine junction, middle enamel development starts by the outgrowth of the bud-like extensions as previously described (Fig. 1, A and B). These extensions increase in length and width and develop into longitudinal rods (Fig. 1C). The apatite crystals in these rods are grouped parallel to the rod axis and are similar in orientation to those of the inner enamel.

At this stage, the ameloblastic cytoplasm protrudes between the longitudinal rods. By intracytoplasmic differentiation of matrix and calcification of the latter, the distal portions of the protruding ameloblasts are progressively filled out so as to form enamel rods running in a different direction. The complicated architecture which results from this sequence is illustrated diagrammatically in Fig. 1, C and D, as well as in Figs. 12, 13 and 14.

Moreover, the rod interrelationships in the middle enamel layer become more intricate because the longitudinal rods, at a distance of 3–4 μ from the enamel-dentine junction give rise to diverging branches (Figs. 1, C and D; 13 and 14). Thus, the architecture of the middle enamel layer becomes progressively outlined. After the longitudinal rods are deposited, the cytoplasmic gaps between them are filled with rods whose courses run at a different angle. The apatite crystals found in the latter rods (Figs. 13, 14) are oriented parallel to the rod long axis diverging strongly therefore, from the apatite crystals of the adjacent longitudinal rods.

In the peripheral part of the middle enamel layer, the longitudinal rods no longer ramify and hence an appearance of parallel rods is gained (Fig. 1D). However, it is noted that the long axes of the apatite crystals are still different in adjacent rods (Fig. 15).

What we call outer enamel layer (Fig. 1D) is formed by fusion of longitudinally running rods over the underlying interposed rods (Fig. 15), giving rise to a continuous enamel cover containing parallel apatite crystals whose longitudinal axes are perpendicular to the enamel surface (Fig. 16).

While the middle enamel layer presents a complicated rod structure, the inner and outer enamel layers consist of a continuous juxtaposition of apatite crystal aggregates with parallel axes.

In 1-year-old rats, the erupted enamel of the lower incisors presents essentially the same rod interrelationship as that described above. But, even in the adult animal,

some variations can be found in the degree of mineralization. This may be explainable by the sequence of apatite crystallization already described during development (Fig. 20). While various intermediate steps can be found, there appear to be two principal variations in the degree of inorganic crystallization in the adult: first, areas of well-developed apatite crystals characterized by complete fusion of the calcifying fibrils; second, areas of unfused calcified fibrils where the reticular appearance of the protein matrix is still visible (Fig. 17). Yet, it is noteworthy that even the unfused calcified fibrils gave an electron diffraction pattern typical of the apatite crystal group.

Unlike the incisors, the molars of the rat are anatomically more comparable to human teeth. On this basis it has been generally assumed that this similarity may also hold at the microscopic level. Such is not the case, however, for molar enamel of the rat exhibits an interrelationship between the rods different from that of human enamel (Figs. 18 and 19). In regions which would appear in the light microscope to consist of parallel-oriented rods, apatite crystal orientation may be at right-angles from one rod to the next. As a result, one rod will exhibit longitudinally oriented crystals whereas adjacent rods will show cross-cut apatite crystals (Fig. 19).

This study of amelogenesis and of adult rat enamel can be summarized diagrammatically as indicating the intergrowth of fibrous protein and apatite crystal by a process that perhaps may be best likened to a petrification (Fig. 20). Furthermore, it allows us to make a statement concerning the outer spatial morphology of well-formed apatite crystals found in enamel. These crystals, which present an elongated rectangular form in longitudinal sections and hexagonal contours in transverse sections, are considered to be hexagonal prisms, in keeping with observations in human enamel described elsewhere (FRANK, SOGNAES and KERN, 1960).

DISCUSSION

The observations suggest that amelogenesis must be conceived of as a continuous process which cannot be divided into distinctly separated phases. The classical concept, based on routine histological examination, generally differentiated between two distinct steps, elaboration of a pre-enamel organic matrix and inorganic crystal deposition, the latter stage of which being referred to as enamel "maturation" (DIAMOND and WEINMANN, 1940; WEINMANN, WESSINGER and REED, 1942; MARSLAND, 1951, 1952). Though it has become traditional to segregate the two phenomena of matrix formation and calcification, it is noteworthy, in retrospect, that SAUNDERS, NUCKOLLS and FRISBIE (1942), utilizing special histological techniques, concluded that calcification of rat enamel matrix progresses gradually from the dentine-enamel junction to the periphery. Additional support for this concept has accumulated more recently, as various new techniques have been applied to this problem, such as X-ray diffraction (TRAUTZ, 1953; HAMMARLUND-ESSLER, 1958), microradiography (DARLING, 1956; HAMMARLUND-ESSLER, 1958; CRABB, 1959), polarizing microscopy (DARLING, 1956; CRABB, 1959), fluorescence and dark-field microscopy (HALS, 1953).

From these several approaches, and our own, it appears that the enamel is first completed along the dentine-enamel junction and from there progresses towards the enamel surface through an alternating pattern, each step involving a combination

of matrix differentiation and calcification. Furthermore, at the ultrastructural level we have noted that even in the same enamel rod, different degrees of matrix differentiation and calcification may exist, both within small sub-microscopic areas as well as along the entire length of the enamel rod.

Comparing amelogenesis and dentinogenesis, it appears that the dentine matrix differentiates as an extracellular product, in which protein fibrils with ultrastructural characteristics of collagen, are elaborated in the intercellular space existing between the amelodentinal junction membrane and the cell membrane of the odontoblast (WATSON and AVERY, 1954; NYLEN and SCOTT, 1958). In contrast, enamel matrix is an intracellular product, which does not exhibit the ultrastructural periodicity of collagen. We have noted that fibrogenesis during the formation of enamel can start in two ways; in part by the diffuse accumulation of protein fibrils scattered within the ameloblastic cytoplasm (Figs. 2 and 3), and in part by the elaboration of more highly organized electron-dense matrix precursors, which in transverse sections assume an ovoid or rounded form, and in longitudinal sections appear as somewhat elongated cords (Figs. 4, 5, 6). According to TEN CATE (1959), similar structures have been noted by FEARNHEAD. These bodies may also be comparable to those which SAUNDERS, NUCKOLLS and FRISBIE (1942) classified as keratohyaline granules on the basis of the histological staining method of Unna. Various stages of matrix differentiation can be seen in these elongated ovoid bodies (Figs. 4, 5, 6). Presumably they are closely associated with protein synthesis. Ultrastructurally, these bodies are very similar in appearance to the electron-dense keratohyaline granules observed in electron micrographs of keratinizing oral mucosa (SOGNNÆS and ALBRIGHT, 1956).

Our ultrastructural observations on amelogenesis indicate that enamel mineralization commences at a very early stage with the deposition of calcium salts in a crystalline state. This concept is in keeping with the conclusion first reached by HOPPE (1862), later reiterated by SCHMIDT (1925), and more recently supported by the findings of HALS (1953), TRAUTZ (1953), WATSON and AVERY (1954) and HAMMARLUND-ESSLER (1958).

It seems quite clear from our non-decalcified sections of calcifying and calcified enamel that apatite crystallization starts specifically on and within the longitudinally oriented protein fibrils of the pre-existing reticulum matrix. The result of this organic-inorganic intergrowth is a petrification of the enamel reticulum suggesting the possibility of an epitactic phenomenon (FRANK, SOGNNÆS and KERN, 1960), though conclusive evidence for the latter concept must await more complete agreement on the nature of the enamel fibrous protein.

It is commonly assumed that a finished enamel rod is produced by one single ameloblast. This theory is doubted by WATSON and AVERY (1954), who think that the enamel rods in rodents are formed by the synergistic activity of different ameloblasts. From our results, it seems that, within the middle enamel layer, the cytoplasm of one ameloblast contributes to the elaboration of more than one enamel rod (Figs. 1 and 13).

According to LENZ (1957, 1958) and QUIGLEY (1959), the ameloblastic cell membrane disappears completely along the lateral side of the ameloblasts. Our

findings suggest that this may be the case only in those zones which are immediately or directly involved in matrix differentiation, whereas below these areas the lateral cell wall of the ameloblasts may be still present.

Concerning the microscopic anatomy of rat enamel, it is interesting to note that the inner and the outer enamel layers are devoid of rod structure and contain a large number of apatite crystal aggregates with parallel axes. These two layers are in continuity with the longitudinal rods of the middle layer and contain similarly orientated apatite crystals. Layers of rods, which run in a different direction and which are laid down later, fill the spaces found between the longitudinal rods. The result of this complicated rod relationship in the middle enamel gives a pattern of alternating rods similar to that first noted by TOMES (1849) and recently described in the electron microscopic study of WATSON and AVERY (1954). At the ultramicroscopical level we have noted, furthermore, that the changes in direction of the rods follow the different orientations of both the protein fibrils and the apatite crystals in adjacent rods. Finally, it would appear that rat enamel, in addition to its complicated over-all microscopic architecture, is also devoid of the rod sheaths and inter-rod substance characteristic of human enamel (FRANK, 1959).

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